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Volume

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DRAFT FINAL REPORT
VOLUME I

DEVELOP A NOVEL BIOMASS CATALYSED PRETREATMENT AND HYDROLYSIS FOR COSOLVENT FUEL BUTANOL AND ETHANOL FERMENTATION

RENEWABLE ENERGY DIVISION

PREPARED FOR:

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Renewable Energy Division
Coal and Alternative Energy Branch
Energy, Mines and Resources Canada
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PREFACE

This is the FINAL Report of research carried out under this Bioenergy Development Program project. This Report describes the status of the research in some detail, and also refers to related research carried out in the laboratory of Professor Morris Wayman at the University of Toronto, as well as the results of collaboration with St. Lawrence Reactors, of Mississauga, Ontario, Professors Chornet and Overend at Universite de Sherbrooke, and others mentioned in the Acknowledgements.

The purpose of this research project was to define a cost-effective process for conversion of wood and certain agricultural crop residues to fuel alcohols. Ethanol and butanol are now widely used as extenders of gasoline, and are particularly valuable as octane-enhancers, and as co-solvents in methanol motor fuel. The research covered by the contract was to explore a particular process in which a mild acid catalyst is used to produce sugars from these substrates for fermentation to these fuel alcohols. The catalytic agent, SO2, is widely available, and 100 years of experience in its use in pulping has solved problems of material handling, corrosion, environmental protection and the like. The multi-stage process under study has a few optional routes, for example SO2 prehydrolysis may be followed by acid or enzyme hydrolysis, and hydrolysis by fermentation. Acid hydrolysis may be carried out in a continuous plug flow reactor such as that installed at St. Lawrence Reactors. Enzyme hydrolysis is a relatively simple and elegant process, but it has economic problems because of the high cost of making the highly efficient enzymes required. Also it is far from clear even yet whether it is best to recover the lignin of the feedstock before or after hydrolysis, the latter being physically simpler, but the lignin may be more useful if recovered before hydrolysis.

In studies made during the period of this contract, a number of highly significant advances were made in the technology and in the science of conversion of lignocellulosic feedstocks to alcohols, sufficient to alter favorably the economics and the prospects for biomass ethanol, and perhaps butanol. The principal ones discussed in this Report are as follows:

1. Pretreatment: SO2 pretreatment has been firmly established both in the laboratory and on a pilot scale as the method of choice for preparation of lignocellulosic feedstocks for further processing into fuel alcohols.

2. Pentose fermentation: We have solved this problem by using new yeasts which we have modified to produce new strains for rapid and efficient fermentation of the pentose component of these feedstocks simultaneously and in the same vessel as fermentation of their normal 6-carbon sugars.

3. Butanol: We have established in our laboratory an excellent new process for butanol fermentation.

4. Record High Ethanol Yields: We regularly produce at least 350 L per dry tonne of these feedstocks, with some yields approaching 400 L/tonne, in alcohol solutions above 5% or more by volume. We believe these figures to be a record.

5. Simultaneous Enzyme Saccharification and Fermentation using Mixed Cultures. By using efficient and rapid cellobiose and pentose fermenting yeasts in mixed cultures during enzyme hydrolysis, we have achieved record levels of ethanol production, 384 L/tonne, from aspen. In this process, unlike in all others, addition of the costly enzyme cellobiase is not required.

6. Novel Bioreactor: We have developed on a laboratory scale a novel continuous dynamic immobilized cell bioreactor in which sugar is fermented in less than 2 hours, and using our new yeast strains, wood hydrolysates are fermented rapidly and efficiently.

7. Enzyme Production: We have in the laboratory a new process for making cellulase enzyme which may be more economic than others now under study.

We believe the advances in science and technology listed above have improved materially the economics and the prospects for industrial conversion of biomass to fuel alcohols.

This Report is intended to reach government research policy decision makers at Federal and Provincial levels, industrial corporations planning entry into the bioenergy field, researchers in other institutions and laboratories, and the interested public generally.

The research is still incomplete, and while we are encouraged by the findings so far, no hard and fast conclusions can be drawn until experience is acquired on a larger scale, preferably in demonstration plants, so the economics of conversion of lignocellulosics to liquid motor fuels can be stated with more confidence.

ABSTRACT

The objective of this Bioenergy Development Program research and development project was to define conditions for carrying out a cost-effective process for the SO2-catalysed conversion of wood and agricultural materials - lignocellulosics - to butanol and ethanol, which are motor fuel extenders, octane enhancers and co-solvents in methanol-gasoline blends. The feedstocks studied under this contract were aspen poplar chips, pine sawmill residues, mixed coniferous wood chips and corn stover. Earlier laboratory results had shown that a small amount of SO2 could be used advantageously compared to any other acid to catalyse hydrolysis of hemicellulose and, after separation of the hemicellulose sugars, also hydrolysis of the residual cellulose. This earlier work had not included fermentation, which may, under unfavorable conditions such as high temperature sulphuric acid hydrolysis, present serious difficulties. The end-products aimed for in this project were butanol and ethanol. Butanol fermentation is difficult either in the laboratory or industrially. When this project began, the pentose sugars formed from the hemicellulose, which are a substantial percentage of the substance of these feedstocks, could not be fermented efficiently to ethanol, and even their efficiency of conversion in butanol fermentation was unclear.

To study these matters we established a high quality anoxic chamber for butanol fermentation, a bench scale (7 L) direct steam reactor for prehydrolysis and hydrolysis of chips, sawdust and other forms of biomass, proper analytical equipment, equipment for treating sugar solutions ahead of fermentation, and extensive ethanol fermentation equipment and procedures. We also collaborated with other institutions and corporations, including St. Lawrence Starch Company, Universite de Sherbrooke, Wenger Manufacturing and others, to prepare on a pilot plant scale stocks of treated fibres for our researches. We also found it beneficial to study fermentation of spent sulphite liquor from 3 different pulp mills, which gave us further insights into problems of fermenting solutions having toxic or inhibitory substances. These investigations also gave us the opportunity to develop quite new yeast strains with special merits for our purposes.

Those of our principal findings which represent significant advances in the science and art of conversion of biomass to fuel alcohols may be listed as follows:

1. Pretreatment: SO2 pretreatment has been firmly established in both laboratory and pilot plant runs as being an effective step in freeing hemicellulose and lignin, and in preparing the cellulose of these feedstocks for either acid or enzyme hydrolysis. This pretreatment has been carried out in continuous equipment in which chips of aspen or of coniferous woods were treated at 100 kg to 1500 kg/hour in 2 quite different kinds of commercially available equipment, the Stake reactor and the Wenger extruder. Some thoughts comparing these equipment types are included in this Report.

2. Pentose Fermentation: When this study began, there was a most serious problem related to ethanolic fermentation, in that the pentose sugars, which constitute a significant proportion of the composition of these feedstocks, could not be fermented to ethanol by common yeast, which ferments only hexose sugars. We have been very fortunate in that we have solved this problem completely, using other yeasts, and modifying them to produce more efficient strains, and defining conditions for optimal use. These new yeasts, which we call tentatively "R strains", are quite different from the wild strains being studied by others. They ferment rapidly and efficiently, with very high yields from both hexose and pentose sugars, including mixtures of these sugars such are obtained by hydrolysis of biomass, and are tolerant of very high concentrations of sugars and of ethanol. We have recently introduced our R yeasts into a continuous large pilot plant installed for this purpose in a large sulphite alcohol plant. A flow sheet of the pilot plant is included in this Report.

   These new fermentation processes mark substantial advances in the science of biotechnology which we fully expect to be applied to industrial practice. Any industrial plant built to produce ethanol from wood sugars will be well advised to use our R strains.

3. Butanol: We have developed and established in our laboratory an excellent process for butanol fermentation. The main difficulty with butanol fermentation arises from microbial intolerance of butanol concentrations much above 2%. This low beer concentration results in high product recovery costs. By extracting the butanol as formed with a good extractant, we are able to achieve improved yields.

4. High Ethanol Yields: We have been successful in converting these feedstocks to ethanol in very high yields, in a relatively simple process. In this process, SO2 prehydrolysis is followed, without any separation of hemicellulose sugars or lignin, by enzyme hydrolysis, and this in turn by fermentation by R yeast. Sugar solutions of above 100 g/L are obtained which on fermentation give rise to alcohol solutions of more than 5% by volume. We have regularly obtained 350 L ethanol or more per dry tonne of feedstock. These are higher values than others have achieved.

5. Simultaneous Saccharification and Fermentation using Mixed Cultures: We have identified a yeast which is a rapid and efficient fermenter of cellobiose. Cellobiose is a product of cellulose hydrolysis whose formation severely inhibits the hydrolytic action of cellulase enzyme. The addition of cellobiase, as is normal practice for those employing enzyme hydrolysis, but which is expensive to make or buy, assists in ameliorating this problem, but this enzyme in its turn is inhibited by the glucose which it forms from the cellobiose. By removing these sugars as formed hydrolysis proceeds much more rapidly and efficiently. A mixed culture of this yeast together with our R yeast when applied along with cellulase to SO2 pretreated aspen resulted in 384 L ethanol per dry tonne of wood in 48 hours, 90% complete in 24 hours. This was a new record for ethanol production from this feedstock, in quantity, concentration and rate. The expense of added cellobiase is avoided completely in this new process. This simplest and most productive of processes is being pursued further, with alternative pretreatments and with other biomass feedstocks.

6. Enzyme Production: We have under development a process for making cellulase enzyme, suitable for in-plant application, based on new principles. In it, aspen wood (and of course other feedstocks can be used) is solubilized in such a way as to make a mixture of oligomers with very little monomer. This solution is an excellent inducer of cellulase produced by Trichoderma reesei, the mould most commonly used for this purpose. We have proposed a pilot scale production run based on this process.

7. A Novel Bioreactor: We have developed a novel continuous dynamic immobilized cell bioreactor which, when loaded with yeast, has fermented sugar solutions in less than 2 hours. When R yeast was used in it, wood hydrolysates were fermented more rapidly and efficiently than in other fermenters described so far. This fermenter is ready for upscaling.

This research and development project has been significant because the advances in science and technology listed above have improved materially the economics and the prospects for industrial conversion of wood and agricultural materials to fuel alcohols. By application of these new findings, economic targets can be met, certainly wherever low cost feedstocks are available, be they sawdust or other forest industries wastes, spent sulphite liquor, straws, cobs, whole grains, or, eventually, the products of energy plantations.

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EXECUTIVE SUMMARY

The objective of this Bioenergy Development Program research and development project was to define a cost-effective process for conversion of wood and agricultural materials - lignocellulosics - to fuel ethanol and butanol. These alcohols are widely used as extenders of, or even substitutes for, gasoline, as environmentally preferred replacements for lead in gasoline, as octane enhancers and as co-solvents in methanol-gasoline blends. The research and development covered by this contract was to explore a particular process in which a mild acid catalyst is used to produce sugars, and from them the alcohols by fermentation, from designated feedstocks, aspen and pine woods and corn stover. The preferred catalytic agent, SO2, is readily available from natural gas or the metallurgical industry, and has long been used in the manufacture of pulp and paper. The 100 years of experience in pulping has provided solutions to problems of materials handling, corrosion, environmental protection, and the like. We began this project based on earlier studies which suggested that this was the most efficient catalyst for this purpose. However, when the project began, there were many unsolved problems related to the best equipment to be used in pretreatment and in hydrolysis of these feedstocks, the possibility of formation of substances toxic or inhibitory to fermentation, how to ferment the 5-carbon sugars, the pentoses which form a large proportion of the substance of lignocellulosics efficiently and in the same vessel and at the same time as the conversion of the hexose sugars which yeasts normally ferment, how to achieve yields of ethanol well above those normally cited, that is how to go from 200-250 L ethanol to 350-400 L ethanol per dry tonne of biomass, how to overcome the very long times required to produce and to use the enzymes which can hydrolyse these cellulosics under gentler conditions than the more widely employed acid hydrolysis, and how to overcome the serious product inhibition in butanol fermentation. We are now able to report successful solutions to these problems.

This Report describes both laboratory and pilot plant scale research and development to study these matters. We have carried out SO2 pretreatment of aspen and coniferous woods in continuous equipment in which chips were treated at 100 to 1500 kg/hour; and have carried out fermentations of pentose-containing wood-derived hydrolysates in a 1200 L continuous fermenter. We have established and operated a unique anoxic chamber for butanol fermentation, a bench scale (7 L) direct steam reactor for prehydrolysis and hydrolysis of chips, sawdust or other biomass forms, the requisite analytical equipment, and extensive equipment and procedures for ethanol fermentation. In addition, we have used the facilities of other institutions and corporations, including St. Lawrence Reactors, Universite de Sherbrooke, Wenger Manufacturing, and others to prepare on a pilot scale stocks of treated fibre for our study. We also

studied fermentation of spent sulphite liquor from 3 different pulp mills, thereby acquiring insight into problems of fermenting highly toxic solutions. By adapting pentose-fermenting yeasts to these solutions, we have developed new yeast strains which confer special benefits upon our fermentations.

The conversion of biomass to fuel alcohols must be carried out in at least 2 stages, in the first of which, pretreatment or prehydrolysis, the hemicellulose, which constitutes 20 to 40% of the raw feedstock and is therefore too much to be ignored, is solubilized and may be separated, if desired, from the residual mixture of cellulose and lignin. Then there are several options: the lignin may or may not be separated by solution in caustic or organic solvents; the cellulose may be hydrolysed by either acids or by enzymes. The technology of the larger scale acid hydrolysis has been intensively studied in collaboration with St. Lawrence Reactors Limited, which has built a pilot plant for this purpose. We have also studied and made significant advances in enzyme hydrolysis. This latter is a most elegant process, without physical or mechanical limitations, but it has economic uncertainties because of the high cost of making the highly efficient enzymes required. In response, we have also been studying, with some success, processes for enzyme production.

The main highlights of the significant scientific and technological advances made during the course of this contract are best summarized in the following list.

1. Pretreatment: SO2 pretreatment has been firmly established in both laboratory and pilot plant runs as being an effective step in freeing hemicellulose and lignin, and in preparing the cellulose of these feedstocks for either acid or enzyme hydrolysis. This pretreatment has been carried out in continuous equipment in which chips of aspen or of coniferous woods were treated at 100 kg to 1500 kg/hour in 2 quite different kinds of commercially available equipment, the Stake reactor and the Wenger extruder. Some thoughts comparing these equipment types are included in this Report.

2. Pentose Fermentation: When this study began, there was a most serious problem related to ethanolic fermentation, in that the pentose sugars, which constitute a significant proportion of the composition of these feedstocks, could not be fermented to ethanol by common yeast, which ferments only hexose sugars. We have been very fortunate in that we have solved this problem completely, using new yeasts, and modifying them to produce more efficient strains, and defining conditions for optimal use. These new yeasts, which we call tentatively "R strains", are quite different from the wild strains being studied by others.

They ferment rapidly and efficiently, with very high yields from both hexose and pentose sugars, including mixtures of these sugars such are obtained by hydrolysis of biomass, and are tolerant of very high concentrations of sugars and of ethanol. We have recently introduced our R yeasts into a continuous large pilot plant installed for this purpose in a large sulphite alcohol plant. A flow sheet of the pilot plant is included in this Report.

These new fermentation processes mark substantial advances in the science of biotechnology which we fully expect to be applied to industrial practice. Any industrial plant built to produce ethanol from wood sugars will be well advised to use our R strains.

3. Butanol: We have developed and established in our laboratory an excellent process for butanol fermentation. The main difficulty with butanol fermentation arises from microbial intolerance of butanol concentrations much above 2%. This low beer concentration results in high product recovery costs. By extracting the butanol as formed with a good extractant, we are able to achieve improved yields.

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5. Simultaneous Saccharification and Fermentation using Mixed Cultures: We have identified a yeast which is a rapid and efficient fermenter of cellobiose. Cellobiose is a product of cellulose hydrolysis whose formation severely inhibits the hydrolytic action of cellulase enzyme. The addition of cellobiase, as is normal practice for those employing enzyme hydrolysis, but which is expensive to make or buy, assists in ameliorating this problem, but this enzyme in its turn is inhibited by the glucose which it forms from the cellobiose. By removing these sugars as formed hydrolysis proceeds much more rapidly and efficiently. A mixed culture of this yeast together with our R yeast when applied along with cellulase to SO2 pretreated aspen resulted in 384 L ethanol per dry tonne of wood in 48 hours, 90% complete in 24 hours. This was a new record for ethanol production from this feedstock, in quantity, concentration and rate.

The expense of added cellobiase is avoided completely in this new process. This simplest and most productive of processes is being pursued further, with alternative pretreatments and with other biomass feedstocks.

6. Enzyme Production: We have under development a process for making cellulase enzyme, suitable for in-plant application, based on new principles. In it, aspen wood (and of course other feedstocks can be used) is solubilized in such a way as to make a mixture of oligomers with very little monomer. This solution is an excellent inducer of cellulase produced by Trichoderma reesei, the mould most commonly used for this purpose. We have proposed a pilot scale production run based on this process.

7. A Novel Bioreactor: We have developed a novel continuous dynamic immobilized cell bioreactor which, when loaded with yeast, has fermented sugar solutions in less than 2 hours. When R yeast was used in it, wood hydrolysates were fermented more rapidly and efficiently than in other fermenters described so far. This fermenter is ready for upscaling.

The main CONCLUSIONS of this work may be listed as follows:

1. Prehydrolysis of biomass employing a small amount of SO2 as catalyst is highly effective as pretreatment ahead of either acid or enzyme hydrolysis. It gives rise to fewer problems in fermentation than other acids, or in using pretreated stock for enzyme production; it can be used in ordinary stainless steel equipment and is easy to carry out on an industrial scale. Several suitable makes of equipment are commercially available.

2. Simultaneous fermentation of pentose and hexose sugars such as are formed on hydrolysis of wood or other biomass, or are present in spent sulphite liquor, can be carried out rapidly and efficiently. Our R strain of yeast, a new strain, is better suited for this purpose than the many other yeasts we have tested. It is suitable for industrial operations.

3. The technology for enzyme hydrolysis of SO2-pretreated biomass is well worked out on the small scale. The process is simple and elegant. The very high yields of ethanol are achieved, in the range of 360 to 390 L/dry tonne of biomass, particularly commend this process.

4. When enzyme hydrolysis is carried out simultaneously with fermentation by a mixed culture of cellobiose- and pentose fermenting yeasts, no cellobiase enzyme is required, the process is rapid and efficent, and the highest yields of ethanol, about 85% of theory, are reached. While further improvements in the technology and economics of this process may be expected when it is practiced on a larger scale, it is highly unlikely that these ethanol yields will be exceeded.

5. Extractive butanol fermentation overcomes product inhibition and produces butanol in higher concentrations and in higher yields than ordinary non-extractive fermentation.

6. A new continuous dynamic immobilized cell bioreactor is good for very rapid and efficient fermentation, and can be used with ordinary yeast for hexose sugar solutions, including molasses, and with R yeast for pentose-containing solutions such as biomass hydrolysates.

7. A new cellulase enzyme production process merits further exploration as an in-plant adjunct to enzyme hydrolysis.

8. THE SCOREBOARD

ALCOHOL YIELDS OBTAINED FROM LIGNOCELLULOSICS

	PINE	ASPEN	CORN STOVER
SO2 PREHYDROLYSIS			
sugars, kg/tonne	219	252	130
ENZYME HYDROLYSIS			
sugars, kg/tonne	717	680	650
ETHANOL			
L/tonne	372	346	388
S.S.F. mixed culture			
L/tonne		384	
BUTANOL SOLVENTS			
L/tonne	292	255	283
THEORETICAL YIELDS			
ethanol, L/tonne	484	470	480
butanol solvents, L/T	345	340	343

PRACTICAL YIELDS, which may safely be expected:
ETHANOL: 350 - 390 L/tonne
BUTANOL SOLVENTS: 250 - 300 L/tonne

The main RECOMMENDATIONS of this study are listed here:

1. That a full assessment of the relative merits for pretreatment of the Wenger and Stake equipment, and perhaps the Sunds, Masonite and other commercially available reactors, be carried out, with wood chips, sawdust, and agricultural materials.

2. That intensive study of acid hydrolysis of cellulose be continued on a pilot plant scale, including residue recycle, cleanup of sugar solutions, etc.

3. Following economic evaluation of the costs of cellulase enzyme in-plant production, a demonstration or small industrial plant to convert sawdust, waste chips or agricultural materials be built, preferably alongside an existing distillation plant or an oil refinery, to use SO2 pretreatment, S.S.F. with single cellulase enzyme and mixed culture as described here, and distillation-dehydration.

4. That more economical in-plant cellulase enzyme production processes be developed, and that this soon move to the pilot plant scale.

5. That lignin separation, preparation and evaluation be emphasized in future research and development programs.

6. That a large prototype of the continuous dynamic immobilized cell fermenter be built, preferably in an existing alcohol plant.

7. That butanol production research based on extractive fermentation be re-established.

SO WHAT?

The advances in science and technology described in this Report have improved materially the economics and the prospects for industrial conversion of wood and agricultural materials to fuel alcohols. By application of these findings, economic targets can be met, certainly where low cost feedstocks are available, be they sawdust, other forest industries wastes, spent sulphite liquor, straws, cobs, whole grains, or, in the longer term, the products of energy plantations. Research and development as described here are not yet complete - perhaps r & d is never complete - and no hard and fast conclusions can be drawn until experience is acquired on a larger scale, preferably in demonstration plants, so that the economics of conversion of wood and other lignocellulosics to motor fuels can be stated with confidence. Nevertheless our findings have led us to the assessment implicit in the CONCLUSIONS listed above.

Our RECOMMENDATIONS urge the early implementation of these findings on the larger, pre-industrial or in some locations industrial scale. The RECOMMENDATIONS also list those areas which have the greatest need for further research, and the greatest promise for improving further the prospects for industrial conversion of biomass to liquid motor fuels.

RESUME

DEVELOP A NOVEL BIOMASS CATALYSED PRETREATMENT AND HYDROLYSIS FOR CO-SOLVENT FUEL BUTANOL AND ETHANOL FERMENTATION

1. INTRODUCTION

Propelled by a vision of increasing demand for petroleum resources and shrinking supplies over the longer term, undeterred by temporary surpluses and lowering of oil prices, there is a perceived need for gasoline extenders and even replacements. In addition, the phasing out of lead in gasoline has led to the recognition of the alcohols as the environmentally preferred octane enhancers. These considerations have led to widespread adoption of alcohols as motor fuel extenders in the United States and to a much more limited extent in Canada, while in Brazil alcohol is used country-wide not only as an extender at about a 20% level, but also to a considerable extent as a replacement for gasoline. In the United States alcohol is supplied from corn-based distilleries, and butanol from petrochemical operations. In Canada, while both these alcohols may find their place in the market, methanol, of which Canada has very large productive capacity, may turn out to be the fuel extender of choice. Methanol has the distinct disadvantage, however, that its use requires a co-solvent to prevent serious performance problems, especially in a cold climate. Either ethanol or butanol are suitable co-solvents. While fuel alcohol in Brazil, the United States and in Canada is made by fermentation of agricultural products, our Canadian resource supply suggests strongly that forest industry wastes, particularly aspen poplar chips and pine sawmill residues, would be the most economical and available feedstock for fuel alcohol plants. Resource studies have also suggested that corn stover, cobs or low grade grains could make a significant contribution to such supply. In the longer term, energy plantations are renewable resources which can supply our need for feedstock. These considerations led to the undertaking which forms the substance of this research and development project: to develop a cost-effective process for production of fuel ethanol and butanol by fermentation of sugars derived by hydrolysis from these feedstocks: aspen, pine and corn stover. Preliminary work has suggested that the use of a mild hydrolysis catalyst, sulphur dioxide, in place of the far more drastic catalysts used by others, would have significant operating and cost advantages over previously suggested, or operated, processes.

All industrial operations to make fermentable sugars from wood have, so far, been based on the fundamental German design of Scholler, in which dilute sulphuric acid is passed through a bed of wood chips at very high temperatures, and the sugar solutions so

obtained are recovered for purification and fermentation. While this process was, under special war-time conditions, operated in Germany and in the United States, all presently operating plants, said to be about 40 in number, are, with one exception, in the Soviet Union. The sole exception is a plant in Brazil. There are a number of pilot plants, notably one in New Zealand, which have adopted this procedure and have reported extensive studies. The results show that corrosion is so serious as to be crippling, the sugar solutions contain toxic compounds which inhibit fermentation, and the yields of sugar are, at best, about 40 to 50% of what could be expected. There is an obvious need for a better process.

The Stake or Iotech autohydrolysis-enzyme processes give good yields of ethanol from hardwoods such as aspen, but were, until now, limited by their inability to accept conifers, and by the expense of the enzymes. There has been, heretofore, no good enzyme production system which would meet the economic requirements of a fuel ethanol process. Enzyme economics are limited by the complexity of the multi-component enzyme system, and its slow production rate. The acid-based processes have so far given poor yields, and serious corrosion problems. The only exception has been the claims of Paszner, not so far confirmed, and even then linked to the inherent costs of a solvent pulping system. In 1985, Wayman and his colleagues at the University of Toronto reported on a new and milder catalyst, sulphur dioxide (10.5, 10.6) and in their hands quantitative yields of sugars were obtained, albeit on a small scale, from hemicellulose and acceptable yields from SO2-catalysed hydrolysis of a variety of biomass feedstocks, including aspen, pine and corn cobs. Comparison with other acids (10.4) showed SO2 to be most effective in sugar yields. This preferred catalytic agent, SO2, is readily available from Natural gas or the metallurgical industry, and has long been used in the manufacture of pulp and paper. The 100 years of experience in pulping has provided solutions to problems of materials handling, corrosion, environmental and health protection and the like. We began this research and development project based on these earlier studies which showed that this is the most efficient catalyst for this purpose.

These earlier studies (10.5, 10.6) gave yields of ethanol and butanol which were calculated from measurements of sugar production, not actually fermented. This left many questions unanswered. There are serious difficulties with both butanol and ethanol fermentation of wood hydrolysates. Butanol fermentation is very difficult because the bacteria which produce the butanol are intolerant of butanol concentrations above about 2%, which results in high costs for butanol recovery. The yeasts universally used to produce ethanol are able to ferment only a limited range of sugars, such as sucrose and the hexoses glucose, mannose and galactose.

In wood and agricultural crop residues, in addition to hexose sugars there is a very high proportion of pentose sugars, 20 to 40% of their substance, which are not fermentable by common yeast, far too much to be ignored. Since a good hydrolysis process will produce a large amount of these pentose sugars, there was, at the start of this research and development project, a great need for a yeast that would ferment pentoses in high concentrations rapidly and efficiently, as well as a better process for butanol fermentation and recovery. When the project began, there were many other unsolved problems: the best equipment to use in pretreatment and hydrolysis of these feedstocks; the possibility of formation of substances toxic or inhibitory to fermentation; how to ferment these 5-carbon sugars, the pentoses which form so large a proportion of the substance of lignocellulosics efficiently and in the same vessel and at the same time as the conversion of the hexose sugars which the yeasts normally ferment; how to achieve yields of ethanol well above those usually cited, that is how to move from 200 - 250 L ethanol to 350 - 400 L ethanol per tonne of biomass; and how to overcome the very long process times required to produce and to use the enzymes which can hydrolyse these cellulosics under gentler conditions than the more widely employed acid hydrolysis. We are now able to report successful solutions to these problems. This Final Report describes the methods used and the results obtained in this research and development project, it draws CONCLUSIONS from those findings and makes RECOMMENDATIONS particularly relevant to advancing the prospects for conversion of renewable resources to fuel alcohols to pre-industrial or industrial operations.

2. OBJECTIVES

2.1 To establish on a bench scale optimal conditions of a new and relatively simple process for fermentation of co-solvent butanol and ethanol based on SO2-catalysed pretreatment and enzyme or acid hydrolysis at high solid/liquid ratios to make fermentation substrate from three biomass feedstocks.

2.2 The feedstocks to be studied are pine sawmill residues, aspen poplar chips and corn stover.

2.3 To ferment and obtain yields of butanol and ethanol from these feedstocks.

2.4 To develop conceptual plant designs, calculate the economy of these processes, and report the results.

3. METHODS

In this research and development project, we have regarded it as of utmost importance that the principal criterion of the success of any step in the process, be it prehydrolysis, hydrolysis, lignin separation or fermentation, be the efficiency and rate of production of end-product alcohols, which must be used for any plant design and economic calculations. Our objective is not to make sugars which may be unfermentable.

3.1 Experimental Design

The experimental design followed in this work has been to carry out simultaneous studies of the butanol and ethanol fermentation processes, the analytical methods and the SO2 prehydrolysis; these being followed by optimization of enzyme hydrolysis of the SO2 prehydrolysed feedstocks and fermentation of the resulting sugar solutions; and then studies of SO2 hydrolysis and lignin recovery. Each of these involved different equipment and different methods, and the researchers in this group accept responsibility for different phases of the research. They have worked in four areas: butanol fermentation; ethanol fermentation; hydrolysis; and equipment and analytical. Some of the equipment used was purchased or designed and built especially for this project, including the direct steam 7 L reactor, the butanol anoxic (anaerobic) fermentation chamber and some of the equipment used to prepare hydrolysates for fermentation. The feedstocks were obtained from sawmills, industrial sources or a farm. Pilot plant runs were carried out in collaboration with St. Lawrence Reactors Limited, the Universite de Sherbrooke, Wenger Manufacturing, Georgia-Pacific and others. We have carried out SO2 pretreatment of aspen and coniferous woods in continuous equipment in which chips were treated at 100 to 1500 kg per hour; and have carried out fermentations of pentose-containing wood-derived hydrolysates in a 1200 L continuous fermenter. We have studied fermentation of spent sulphite liquor from 3 different pulp mills, thereby acquiring insight into problems of fermenting highly toxic solutions. By adapting pentose-fermenting yeasts to these solutions, we have developed new yeast strains which confer great benefits upon our fermentations. The methods used are described in detail below, or in the publications which accompany this Final Report (listed in Section 10).

3.2 Butanol Fermentation

Our earlier methods used for butanol fermentation are described in the paper by Wayman and Yu, 1985 (10.9) and in the Ph. D. Thesis of Dr. Shiyuan Yu (10.15) The new butanol fermentation method (10.8, 10.14) includes these new elements: a large anoxic (anaerobic) fermentation chamber well supplied with internal processing equipment; extraction during fermentation with dibutyl phthalate, an excellent extractant which also apparently stimulates the growth of the bacteria; recycle of the bacteria from one fermentation to the next; very high inocula; and the selection of the most suitable strains, mostly Clostridium acetobutylicum P262, provided by Professor Woods, of South Africa. A paper describing this fermentation (10.14) has the details of this method, which is summarized as follows:

A procedure for extractive butanol fermentation was established. In preliminary trials, dibutylphthalate was selected as the best extractant. A 1:4 ratio of medium to extractant proved to be the best ratio. The best nutrient level was 2 x Monot's nutrients. Fermentation was carried out in 250 mL Erlenmeyer flasks, in the anoxic chamber under a nitrogen-hydrogen atmosphere. Medium including the test hydrolysate or other substrate having a fermentable sugar content of about 80 g/L was inoculated with Clostridium acetobutylicum P262, suitably prepared, such that the cell density in the medium was 6 to 8 g/L. Four volumes of extractant were added, and the flasks were put on a gentle horizontal shaker within the anoxic chamber, and held at 35 C for 24 hours. At 24 hours, samples were withdrawn from the aqueous layer and analysed for residual sugar and for the solvents, butanol, acetone and ethanol. The cells from the aqueous layer were centrifuged and re-utilized for fermentation in fresh medium. This procedure was repeated several times. Butanol in the extract was recovered into water by extraction, and the concentration of solvents in the aqueous extract was determined. Analysis was by GC.

Substrates used in this procedure included pure sugars, used to establish the method, and hydrolysates of aspen, pine and corn stover.

3.3 Ethanol Fermentation

The development of methods for fermenting pentose sugars and hydrolysates containing both hexose and pentose sugars are described in a series of papers which are in Section 10 (10.1, 10.11, 10.12, 10.13, 10.14, 10.15, 10.16,10.17, 10.19, 10.21). The methods began by selecting certain yeast species belonging to Candida shehatae and Pichia stipitis, and manipulating these wild yeasts in order to develop from them new strains, which we call "R" strains, which are highly efficient in fermenting mixtures of hexoses and pentoses, with a high rate of fermentation, and tolerant of very high sugar concentrations and alcohol concentrations. The methods used are best described in Dr. Yu's Ph.D. thesis and in (10.16). Research now under way has established that these "R" strains are indeed new, man-made organisms, distinct from the wild type not only in performance, but also in genetic composition. Preliminary results confirm this fundamental difference, and investigation of the genetics and taxonomy of these new yeasts (or new yeast?) is proceeding at the University of Western Ontario and at the USDA Northern Research Laboratory, Peoria. Thus far, the yeasts are not identical with any known species.

The only thing different about the fermentation method used to ferment sugars or hydrolysates with these new yeasts is that they require some air: they are not happy with either aerobic or anaerobic fermentation, so we use what we call "semi-aerobic" fermentation, in which a controlled supply of air is fed to the fermenter. We also use much higher yeast concentrations than is customary in laboratory work, about 1 to 2% by (freeze dried) weight in the medium; and we recycle the yeast from one fermentation to the next, recovering the yeast for re-use by centrifuging.

Candida shehatae R is being used in a 1200 L continuous fermentation pilot plant built for the purpose of testing the performance of this yeast in the large sulphite alcohol plant at Georgia-Pacific, Bellingham, Washington. A flow sheet of the pilot plant is at 10.22. In spite of the fact that the spent sulphite liquor substrate contains a relatively high concentration of inhibitors, about 60% of the xylose has been consumed. While this is below expectation, it may meet the objectives of the Company, which are related to desugaring and purification of lignin. The operation of the pilot plant is continuing, and it is expected that improved utilization of xylose will be achieved.

Recently, a novel continuous dynamic immobilized cell fermenter has been designed and built, described in detail in (10.3) and (10.20). In this we have fermented sugar solutions, wood hydrolysates and spent sulphite liquors, with excellent results discussed in Section 4.7.

3.4 SO2 Prehydrolysis

Nearly all of our prehydrolysis laboratory work is now carried out in a direct steam 7 L reactor, in which the steam pressure can be varied from about 50 to 200 p.s.i.g., a range which suits our process requirements exactly. We built this reactor for this purpose. Processing conditions vary somewhat with the feedstock, but in general a solution containing 2 to 3% SO2 based on fibre is added to the chips or sawdust, at 50% moisture, and mixed in. The reactor is then closed and steam at 50 to 75 p.s.i.g. is admitted for the required length of time, usually 20 to 30 minutes. The steam pressure is then relieved, the reactor is opened and the product removed. The prehydrolysed biomass may then be washed, or it may be extracted with NaOH or with organic solvents to recover lignin, or it may be treated directly with enzymes to hydrolyse the residual cellulose.

If the hemicellulose sugars are washed out following this prehydrolysis, they are analysed by Y.S.I. Glucose Analyser, for Total Reducing Sugars and by HPLC. The presence of oligomers is determined by carrying out a post-hydrolysis with 4% sulphuric acid at 121 C for 1 hour.

Large scale prehydrolysis runs have been carried out in the Stake reactor at the Universite de Sherbrooke, with the cooperation of Professor Chornet and Overend. These have used aspen chips and mixed coniferous wood chips, first with steam only (autohydrolysis), and then with gaseous SO2 at the rate of 2 to 2.6% on wood. The chips were processed at the rate of 1500 kg/h, which is an industrially acceptable rate. The procedures are described in the joint publication (10.18). The results of this highly successful run are reported in Section 4.3.

A pilot scale run, 100 kg/h, was carried out in a Wenger extruder installed at the BioHol pilot plant in Toronto. This extruder, which is very widely used in the food industry for "cooking" various prepared foods, was employed by Morris Wayman at the Wenger pilot plant in Kansas 6 years ago to pretreat aspen chips from Ontario, and from Alberta. The autohydrolysed chips were then enzyme hydrolysed with excellent results, over 90% of the cellulose being converted to glucose. At that time we knew little about fermentation of these solutions, especially pentose-containing solutions. In the interval, the Wenger equipment was used by BioHol with sulphuric acid, with indifferent success, and its merits were lost track of. However, this equipment has now been revitalized. We ran aspen and corn stover through it, without and with SO2. The product is in a very fine fibrous form, and is being processed further in the laboratory.

3.5 Enzyme Hydrolysis

To hydrolyse the residual cellulose following SO2 prehydrolysis, enzymes are employed. The prehydrolysed biomass is treated with the enzymes directly, without washing, that is the hemi sugars are present. Since the prehydrolysis has been carried out in the absence of much water, enzyme hydrolysis is performed in a high solids state, almost like a solid state fermentation. The action of the enzymes is assisted by gentle agitation, in turn assisted by the presence of stainless steel balls, and contact is assisted by a surface active agent Triton X100. Until quite recently, the enzymes used were a mixture of cellulase, beta-glucosidase and hemicellulase for aspen (3E enzyme), and these are supplemented by pectinase and glucoamylase (5E enzyme) for the pine and corn stover. In addition, certain promoters are used, these being Vitamin B12 and trace elements. All of which illustrates the complexity of enzyme hydrolysis. The amount of enzyme required for good yields in a reasonable time is quite high when the solids is above about 5%, amounting to 5 to 15% on dry fibre. The time required for 90% yields may be 48 hours.

3.6 Simultaneous Saccharification-Fermentation

By carrying out the fermentation simultaneously, or almost simultaneously, with the enzyme saccharification, we have succeeded in simplifying this process considerably, and with much benefit. After 2 to 6 hours at temperature, 45 to 50 C, this almost solid mixture is cooled to about 30 C and the yeast is added directly to it, and in 12 to 18 hours, fermentation is complete. In principle, it can all be done in a single vessel, and completed in 24 hours.

Recently we have been able to make a further quite important advance, using cellulase enzyme only and eliminating the need for the other enzymes. This single enzyme hydrolysis has been quite effective when we have employed a mixed yeast culture consisting of a cellobiose-fermenting yeast (10.17) and our pentose-fermenting yeast. The technique is described in (10.1). The rate of enzyme hydrolysis of cellulose by cellulase is controlled by the formation of cellobiose, which acts as a product inhibitor, a phenomenon common to essentially all enzyme kinetics. By adding cellobiase (beta-glucosidase), the inhibition is removed in part, but the action of the cellobiase is in turn inhibited by the glucose which it forms from the cellobiose. By adding this mixed culture, which effectively removes the two inhibitors as soon as they are formed, by fermentation to ethanol, the rate and efficiency of enzyme hydrolysis are much improved.

3.7 Acid Hydrolysis

The procedures and methods used for SO2 hydrolysis on the bench scale are very similar to those used for prehydrolysis (Section 3.4, and (10.4)), but the amount of SO2 used is generally less, and the temperature is higher, steam pressure being about 190 to 200 p.s.i.g. It is necessary to carry this out with more than one short-time SO2 treatment to prevent sugar destruction. The relationship between pH, time and temperature for optimal sugar recovery is yet to be fully determined.

Pilot scale runs of acid hydrolysis have been carried out at the St. Lawrence Reactors pilot plant, and are described in (10.7). The acid catalyst has been HCl, time about 3 to 6 minutes and temperatures about 190 C to 220 C. In these runs, a multi-pass strategy has been used, with cellulose hydrolysis in the range of 30 to 50% in the first pass. Lower yields are obtained in the second pass. The sugars produced in these pilot plant runs have been fermented in the laboratory, with results discussed in Section 4.3.

3.8 Lignin Recovery

Lignin may simply be recovered following hydrolysis, where the residual solid, which can be removed by simple filtration, is about 90% lignin, or material which tests as lignin. We have some concern about whether this lignin, which is highly condensed by the action of the acid at high temperature, is suited for much higher value than fuel. We therefore have studied recovery of lignin following prehydrolysis. Any one of several alternative procedures may be used: the hemi sugars may be washed from the residual fibre by hot water, and the lignin then extracted with dilute sodium hydroxide and the lignin recovered by acidification; alternatively, the extraction of the fibre may be carried out with sodium hydroxide, a procedure which dissolves both lignin and hemicellulose sugars, and then upon acidification the lignin precipitates, leaving the hemi sugars in solution; or the lignin may be recovered by solvent extraction. All of these have been studied here. We have studied solvent extraction of lignin from the prehydrolysed fibre using a variety of solvents, specifically ethanol, acetone, diethylene glycol, ethylene glycol, ethyl acetate and fusel oil, the last of these being obtained from the alcohol plant of St. Lawrence Starch. Of these, extraction with 70% ethanol at 190 C was most effective in recovering lignin in high yield. In consequence, a larger scale preparation of lignin solvent extracted from aspen wood HCl-pretreated in the St. Lawrence reactor was carried out at Ontario Research Foundation. The resulting lignin is being evaluated by several potential buyers, and the residual fibre is being studied here for hydrolysability and fermentability.

3.9 Analytical

The equipment used for analysis includes a Waters Sugar Analyser HPLC System, a Y.S.I. Glucose Analyser, a Hewlett Packard Gas Chromatograph, a UV-Visible Spectrophotometer, and sundry auxiliaries. The GC is used for the alcohols and solvents, and the HPLC for the sugars. In addition we analyse for Total Reducing Sugars by DNS or Nelson Somogyi. A great deal of time was spent over a period of 2 months attempting to put into service a new experimental column developed for sugar analysis by Waters. We obtained the best sugar separation we have ever seen, but unfortunately the column was not stable, and became unusable after half a dozen analyses. With regret, we reverted to the BioRad HPX-87P column for routine analysis.

Summative analysis of feedstock is based on the Klason method, in which the raw material is treated at 20 C with 72% H_2SO_4 for 2 hours, and the resulting carbohydrate solution is diluted to 4% H_2SO_4, and heated at 121 C for 1 hour. The resulting solution of monomeric sugars is neutralized and analysed by HPLC. From the results, the original composition of the feedstock is calculated.

3.10 Cellulase Enzyme Production

We have a process for making cellulase enzyme, still on a laboratory scale, which is suitable for in-plant application, based on new principles. In it, aspen wood or other feedstock is solublilized to a mixture of oligomers with less than 5% monomer. This solution is an excellent inducer of cellulase production by Trichoderma reesei. We usually add some lactose, a milk product, as co-inducer. The amount of feedstock which needs to be diverted from hydrolysis to enzyme production is now about 5 to 8%, but we hope to decrease this.

4. RESULTS AND DISCUSSION

4.1 Butanol Fermentation

In addition to fermentation of pure sugars and mixtures, we have fermented prehydrolysates and complete hydrolysates of aspen, pine and corn stover to the mixture of solvents, butanol, acetone and ethanol which is the product of the butanol fermentation. These butanol fermentations were performed as described in Section 3.2, on hydrolysates prepared by SO2 prehydrolysis - enzyme hydrolysis, the procedure described in Section 3.5, (10.10)

With aspen hydrolysates, the sugar concentration was 78 g/L, and after 24 hours fermentation this was 95% consumed, the product concentration being 24.7 g/L, the yield being 0.33 g solvent/g sugar

consumed. In successive 24 hour additions of hydrolysate solution with bacterial recycle as described, yields obtained were 0.31 and 0.34 g solvent/g sugar consumed. The hydrolysis process resulted in 64.6 g total sugars/100 g aspen wood. Total solvent production was 21.3% on wood, or 266 L/tonne of aspen (dry basis), an excellent product yield.

Yields of butanol solvents obtained with pine and with corn stover in similar procedures were 293 and 283 L/(dry) tonne of feedstock. These are very good yields (10.10 for more details). The main deficiency of this work is that the extractant chosen, dibutyl phthalate, does not have the best properties. We would prefer a solvent with a higher or a lower density, thereby permitting the use of more concentrated sugar solutions.

4.2 Ethanol Fermentation

Aspen wood contains 44% cellulose and 27% hemicellulose, mainly xylose, while pine wood contains 44% cellulose and 20% hemicellulose, mainly mannose, a hexose. Using the SO2-prehydrolysis - enzyme hydrolysis procedure (Section 3.5) sugar concentrations of 110 g/L were obtained, which on fermentation gave 41 g/L ethanol. This is 27% ethanol on wood, or 346 L/tonne (dry) aspen. This is a yield of 73% of the theoretical maximum (475 L/tonne). (Note that the theoretical yield for aspen = cellulose sugars x .51 + hemicellulose sugars x .465, which = 475 L/tonne.) This 73% yield is after all losses in prehydrolysis, hydrolysis and fermentation.

Results of fermentation of hydrolysates of pine and of corn stover gave yields of 372 and 388 L/tonne of dry feedstocks. Like those on aspen, these are very good yields.

By combining enzyme hydrolysis with fermentation, that is, operating in a simultaneous saccharification-fermentation mode, and using the mixed culture described in Section 3.6, we have obtained with SO2-prehydrolysed aspen, 384 L ethanol/tonne, 81% of theory. This is a new record for production of ethanol from aspen (10.1). This process, which gives the highest yields yet requires the simplest equipment, is being developed further, with other feedstocks and with those variations which may further improve productivity.

The use of the new CDIC fermenter has offered further opportunities for improvement of the process for conversion of biomass to ethanol (10.3, 10.20, and Section 5). This simple fermenter permits very rapid fermentation, and higher efficiencies in ethanol production. It has the potential of being operated as a bioreactor, to convert in one continuous operation biomass SO2-prehydrolysed in the Wenger cooker to ethanol.

4.3 SO2 Prehydrolysis

SO2 prehydrolysis has been carried out in small laboratory reactors (30 mL), the 7 L direct steam reactor, the St. Lawrence Reactors high shear continuous plug flow reactor, The Stake reactor at the Universite de Sherbrooke and in the Wenger extruder. The results are consistently favourable. (10.2, 10.4, 10.5, 10.6, 10.18).

Pine wood on prehydrolysis with 3% SO2 on wood, 30 min, 75 psig steam in the 7 L direct steam reactor resulted in 19.82 % of hemicellulose sugars on wood, being 91.75% of the theoretical yield. Spruce wood in the SLR reactor, using HCl at 88 psig for 6 min gave the same yield. The shorter time required in the SLR reactor is partly due to the somewhat higher temperature (166 compared to 160 C) and partly to the structure of the reactor which, because of its high shear, is inherently more efficient. In the direct steam reactor in our laboratory there is a problem of temperature uniformity, and internals have now been fabricated which, it is hoped, will overcome this problem, permitting shorter reaction times.

Aspen wood in the small laboratory reactors resulted, on SO2 prehydrolysis, in 25.2% hemicellulose sugars, which is 90% of theory, In the 7 L direct steam reactor, the yield of hemicellulose sugars from aspen chips was 92% of theory. These yields, in the 90 - 92% range, can be considered to be satisfactory.

The results of the runs at the Universite de Sherbrooke are described in detail in (10.18). Although these were the first runs made with SO2 on the large scale, which involved some educated guess work of the part of the professors involved, 89% of the hemicellulose sugars were recovered for fermentation in the 2 best runs, and in the best run, 95% of these were as monomers. The products were then enzyme hydrolysed and fermented, with excellent results.

4.4 Enzyme Hydrolysis

All three feedstocks have been SO2-prehydrolysed as described in Section 4.3 and the prehydrolysed biomass has been treated with enzymes, according to the procedure described in Section 3.5. For wood hydrolysis, we expected the 3E enzyme to be quite adequate, and it turned out to be so for the aspen, but for the pine the 5E enzyme had distinct advantages in yield. The 5E enzyme was required for the corn stover, which has starch content.

The yield of sugar obtained by the 3E enzyme hydrolysis of aspen was 95.6% of that theoretically possible. This was achieved in 18 hours at 50 C at pH 4.8. On fermentation, this gave 360 L of ethanol/tonne aspen.

With pine, the same procedure gave only 60% yield of sugars, that is 301 L/tonne. By doubling the amount of enzyme, the yield was raised to 89%, and by using the 5E enzyme this was further increased to almost 100%. However, in both these latter cases the time of enzyme treatment was prolonged from 18 hours to 36 hours. It is apparent that pine is more difficult to hydrolyse by enzymes than aspen. With corn stover, employing the 5E enzyme, the yield of sugars freed in the hydrolysis was 89% of the theoretical.

Enzyme hydrolysis of the coniferous woods SO2-prehydrolysed in the Stake reactor at the Universite de Sherbrooke resulted in total sugar recovery of about 70% on wood, which upon fermentation gave ethanol at 7% by volume, at 372 L ethanol/tonne (10.18).

4.5 Acid Hydrolysis

We are carrying out SO2 hydrolysis on prehydrolysed fibre, working with all 3 feedstocks. Our targets are those established by the results reported in our earlier papers (10.5, 10.6), in which cellulose SO2 hydrolysis was reported as 84.3% of theoretical yield with pine, 68.7% with aspen and 66.2% with corn cobs, while for all these feedstocks the SO2 prehydrolysis yields were well over 90%.

It is becoming increasingly apparent that SO2 hydrolysis as we carry it out must be done in several successive treatments, 2 to 4, with removal of sugars following each treatment. This is an awkward procedure to carry out in small reactors which are heated indirectly, but becomes feasible in the direct steam reactor, where temperature rise is very fast and time at temperature can be closely controlled. The prospect is that 3 treatments will be needed, with 1 - 2% SO2 in each, at 195 C for 3 to 6 minutes each.

The results of researches carried out in the SLR pilot plant are described in (10.7). At this point, it seems probable that targets for the first pass will be reached easily, but there is less certainty about the second pass. Probably only 2 passes would be necessary in the continuous plug flow reactor because of its high shear character, which leads to good reaction contact. There is no difficulty in fermenting the sugar solutions from HCl hydrolysis of pine or aspen, but spruce hydrolysate seems to give rise to some problem. The HCl prehydrolysates, however, all contain inhibitors, which are removed upon steam stripping, whereupon fermentation is successful.

This is a very active area of work both in the laboratory and in the SLR pilot plant, and there is still a substantial gap between our performance and our targets. A great deal of effort is being devoted to closing the gap.

4.6 Lignin Recovery

As explained in Section 3.8 above, we have investigated a variety of methods for recovery of lignin from prehydrolysed feedstocks. The 2 methods which appear to have most promise are alkaline extraction followed by acidification, and solvent extraction at high temperature with 70% ethanol. Samples of alkaline lignin from the laboratory and from the SLR pilot plant have been delivered to Ontario Paper Company for conversion to vanillin, and the results at this time are encouraging. Other uses of lignin are also being explored. Solvent extraction lignin, prepared at Ontario Research Foundation as described in Section 3.9 is also being evaluated, but no results are available as yet. Solvent extraction has the advantage that it does a more complete separation of lignin than alkaline extraction, that is, recovery rates are higher and the residual cellulose is purer, but the conditions are much more drastic and the lignin more condensed, which raises questions about its use for values above fuel value.

In this research and development project, in spite of the obvious desirability of recovery of lignin in a form suitable for high value uses, and some have maintained that the economy of the process may depend on a good market for lignin, a view which we shared at the beginning of this project but do not share now because of the substantial progress we have made in strategies of hydrolysis and fermentation, we have in fact concentrated upon the substance of the contract which did not emphasize lignin recovery, and our only significant advances have been in defining conditions for alkaline and solvent extraction. This is an area of work which now needs a clearer focus and more attention.

4.7 The Continuous Dynamic Immobilized Cell Fermenter.

Results with the CDIC fermenter in producing ethanol rapidly and efficiently have been quite dramatic (10.3, 10.20). This fermenter, which was demonstrated at the Sixth Canadian Bioenergy R&D Seminar, Vancouver, Feb 15-18, 1987, has the significant advantage that the yeast is fixed in place, making unnecessary the large expense of providing centrifuges for yeast recycle. With ordinary bakers' yeast it ferments sugar solutions in less than 2 hours, and molasses in less than 4 hours. When loaded with cells of our R yeast, it ferments steam stripped aspen or pine prehydrolysates in 4 to 5 hours, and a pine wood hydrolysate also in 4 hours. Steam stripped softwood spent sulphite liquor takes somewhat longer, 7 hours, but this is of course much less than the 12 to 24 hours normally required. This fermenter also can be used as a bioreactor, in which enzymes may be immobilized. This is discussed in Section 5, Plant Design and Economics.

5. PLANT DESIGN AND ECONOMIC ANALYSIS

At the Sixth Canadian Bioenergy R&D Symposium, the following were put forth as the values of ethanol to an oil refiner, in c/L:

With oil at, $/bbl	25	14
(i) as 10% in gasoline		
with lead	27	16
no lead	32	20
(ii) as 3% ethanol with 5% methanol, methanol at 15 c/L		
with lead	42	15
no lead	58	26

These figures represent reasonable economic targets that any industrial fuel ethanol plant must meet. The value of ethanol at $14/bbl oil is obviously a very difficult target to meet as long as lead is used in gasoline, but as the concept of ethanol as the environmentally preferred octane enhancer becomes adopted here, as it has in the U.S., the situation eases considerably, especially when the ethanol is to be used in methanol-ethanol blends. It is highly likely that the process to be described here would now meet the economic criterion for no lead in methanol blends with no difficulty, and perhaps also as 10% in gasoline with the price of oil in the $16 to $18 range. When lead is used to raise octane, oil would have to sell for about $18/bbl for this process to be economically successful in methanol blends, and $21 to $22/bbl for it to be successful when the ethanol is to be used as a gasoline extender at a 10% level. Many newer vehicles demand lead free gasoline; the prospects for the rate of rise in the price of oil are clouded to say the least, but the price surely will rise. In what follows we have arrived at a price - not cost, that is we have included acceptable returns on investment - of 23c/L. That surely should find its place in the motor fuel industry.

Based upon our laboratory and pilot plant results, we propose the following plant design:

Aspen or pine wood in the form of chips or sawdust or grain or straw, stover or other agricultural residues has its moisture adjusted to 40 to 60%, preferably mid-range. This is easy to do except with agricultural residues like straw or stover, which may require some steaming to open up dried-out tissues. Straw and stover are very bulky, resulting in materials handling problems which can be overcome by pelleting.

The biomass so prepared is fed to a Wenger extruder, to which gaseous SO2 is admitted at 2 to 3% on (dry) fibre, depending upon the particular feedstock. The use of SO2 may be optional for some of these feedstocks. The Wenger is operated at 1200 psig (8 MPa) 225C, 45 seconds. Optimum conditions vary slightly with different feedstocks and moisture content.

The product of the Wenger is a fibrous mass, lacey and very finely divided, highly suitable for further processing. It is cooled by the addition of an equal weight of water, and delivered to the CDIC bioreactor. The CDIC bioreactor has been loaded with cellulase enzyme, produced in-plant, and with a mixed culture of a cellobiose-fermenting yeast and our R yeast. It is maintained at 38C and the residence time is adjusted for each feedstock, in the range of 12 to 24 hours.

The effluent from the CDIC bioreactor is filtered to recover lignin, and then passes to the beer still, where the ethanol is stripped out. The ethanol is rectified and dehydrated in a conventional system.

In addition to standard utilities and instrumentation, the plant includes facilities for preparation of cellulase enzyme. This is based on the use of a small proportion of the feedstock diverted for this purpose, dextrinized in such a fashion as to produce very little monomer, used as an inducer with one of the better strains of Trichoderma reesei. The use of some lactose in the form of whey or other milk product may be desirable. The amount of feedstock is quite small since the enzyme is immobilized, and should not amount to more than 2 to 5%. Wenger-treated inducer should not require as long a retention time as other products. It does not need to be washed ahead of this fermentation.

The economics for an industrial plant with a capacity of 20 million L ethanol per year follow:

Plant Capacity, 20 million L ethanol + 10,000 tonnes lignin per year

Income: $4.6 + 3.0 = 7.6 million

	'000	
1. Plant Capital		
buildings and land	800	
pretreatment	2,000	
enzyme production	1,400	
CDIC SSF	1,000	
filter	300	
distillation-dehydration	1,500	
utilities, controls	1,000	
Total Plant Capital	8,000	= 40c/L
2. Capital Service		
amortization	800	
interest, 12%	950	
interest, construction	500	
interest on working capital	150	
Total Capital Service	2,400	= 12c/L
3. Operating Cost		
maintenance	400	
labour	500	
chemicals	250	
energy	300	
wood	750	
Total Operating Cost	2,200	= 11c/L
4. Ethanol Selling Price		23c/L

5. Investment = Plant Capital + Working Capital
   = $8.1 + 2.2 = 10.3 million

6. Sources of Funds = Owner of Feedstock + Owner of Technology
   + Marketer, e.g. refiner

7. Sales of Lignin: this analysis permits sales of 10,000 tons of lignin per year, which is considered modest since Western Europe sells 1,000 tons per day of sulphited lignin; at 15 c/lb = 33c/kg, which is about half the median European price.

6. CONCLUSIONS

1. Prehydrolysis of biomass employing a small amount of SO2 as catalyst is highly effective as pretreatment ahead of either acid or enzyme hydrolysis. It gives rise to fewer problems in fermentation than other acids, or in using pretreated stock for enzyme production; it can be used in ordinary stainless steel equipment and is easy to carry out on an industrial scale. Several suitable makes of equipment are commercially available.

2. Simultaneous fermentation of pentose and hexose sugars such as are formed on hydrolysis of wood or other biomass, or are present in spent sulphite liquor, can be carried out rapidly and efficiently. Our R strain of yeast, a new strain, is better suited for this purpose than the many other yeasts we have tested. It is suitable for industrial operations.

3. The technology for enzyme hydrolysis of SO2-pretreated biomass is well worked out on the small scale. The process is simple and elegant. The very high yields of ethanol are achieved, in the range of 360 to 390 L/dry tonne of biomass, particularly commend this process.

4. When enzyme hydrolysis is carried out simultaneously with fermentation by a mixed culture of cellobiose- and pentose fermenting yeasts, no cellobiase enzyme is required, the process is rapid and efficent, and the highest yields of ethanol, about 85% of theory, are reached. While further improvements in the technology and economics of this process may be expected when it is practiced on a larger scale, it is highly unlikely that these ethanol yields will be exceeded.

5. Extractive butanol fermentation overcomes product inhibition and produces butanol in higher concentrations and in higher yields than ordinary non-extractive fermentation.

6. A new continuous dynamic immobilized cell bioreactor is good for very rapid and efficient fermentation, and can be used with ordinary yeast for hexose sugar solutions, including molasses, and with R yeast for pentose-containing solutions such as biomass hydrolysates.

7. A new cellulase enzyme production process merits further exploration as an in-plant adjunct to enzyme hydrolysis.

8. THE SCOREBOARD

ALCOHOL YIELDS OBTAINED FROM LIGNOCELLULOSICS

	PINE	ASPEN	CORN STOVER
SO2 PREHYDROLYSIS			
sugars, kg/tonne	219	252	130
ENZYME HYDROLYSIS			
sugars, kg/tonne	717	680	650
ETHANOL			
L/tonne	372	346	388
S.S.F. mixed culture			
L/tonne		384	
BUTANOL SOLVENTS			
L/tonne	292	255	283
THEORETICAL YIELDS			
ethanol, L/tonne	484	470	480
butanol solvents, L/T	345	340	343

PRACTICAL YIELDS, which may safely be expected:
ETHANOL: 350 - 390 L/tonne
BUTANOL SOLVENTS: 250 - 300 L/tonne

7. RECOMMENDATIONS

1. That a full assessment of the relative merits for pretreatment of the Wenger and Stake equipment, and perhaps the Sunds, Masonite and other commercially available reactors, be carried out, with wood chips, sawdust, and agricultural materials.

2. That intensive study of acid hydrolysis of cellulose be continued on a pilot plant scale, including residue recycle, cleanup of sugar solutions, etc.

3. Following economic evaluation of the costs of cellulase enzyme in-plant production, a demonstration or small industrial plant to convert sawdust, waste chips or agricultural materials be built, preferably alongside an existing distillation plant or an oil refinery, to use SO2 pretreatment, S.S.F. with single cellulase enzyme and mixed culture as described here, and distillation-dehydration.

4. That more economical in-plant cellulase enzyme production processes be developed, and that this soon move to the pilot plant scale.

5. That lignin separation, preparation and evaluation be emphasized in future research and development programs.

6. That a large prototype of the continuous dynamic immobilized cell fermenter be built, preferably in an existing alcohol plant.

7. That butanol production research based on extractive fermentation be re-established.

SO WHAT?

The advances in science and technology described in this Report have improved materially the economics and the prospects for industrial conversion of wood and agricultural materials to fuel alcohols. By application of these findings, economic targets can be met, certainly where low cost feedstocks are available, be they sawdust, other forest industries wastes, spent sulphite liquor, straws, cobs, whole grains, or, in the longer term, the products of energy plantations. Research and development as described here are not yet complete - perhaps r & d is never complete - and no hard and fast conclusions can be drawn until experience is acquired on a larger scale, preferably in demonstration plants, so that the economics of conversion of wood and other lignocellulosics to motor fuels can be stated with confidence. Nevertheless our findings have led us to the assessment implicit in the CONCLUSIONS listed above.

NOTE:

Figures and Tables are numbered with 4 or 5 digits, of which the first 3 or 4 refer to the number of a publication in Section 10, and the last refers to the number within that publication.

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10. PUBLICATIONS

10.1 S.R.Parekh and M. Wayman, Recent advances in xylose and cellobiose fermentation, Proceedings, Sixth Can. Bioenergy R&D Symp, 1987.

10.2 M. Wayman, S.R. Parekh and C. Seagrave, SO2 pretreatment for high yield ethanol production, Sixth Can. Bioenergy R&D Symp. 1987.

10.3 S.R. Parekh and M. Wayman, A new continuous dynamic immobilized cell fermenter, Sixth Can. Bioenergy R&D Symp., 1987.

10.4 M. Wayman, Comparative effectiveness of various acids for the hydrolysis of cellulose, Capter 15 in R.A. Young, R.M. Rowell, eds. Cellulose: Structure, Modification and Hydrolysis, Wiley, New York, 1986.

10.5 M. Wayman, A. Tallevi and B. Winsborrow, Hydrolysis of biomass by sulphur dioxide, Biomass 6, 183, 1984.

10.6 M. Wayman, Hydrolysis of biomass by sulphur dioxide 2. recalculation of ethanol yields including pentose fermentation, Biomass, in press.

10.7 P. Assarsson, M. Holysh, A. Tallevi, M. Wayman and S. Parekh, Acid hydrolysis of lignocellulosics for alcohol production, Chem. Eng. Annual Conference, October 1986.

10.8 M. Wayman, G. Husted, J Santangelo, and S. Levy, Butanol by extractive fermentation using fluorocarbons, Proceedings, VI Intern. Conf. on Alcohol Fuels, 2, 234, 1984.

10.9 M. Wayman and S. Yu, Acetone-butanol fermentation of xylose and sugar mixtures, Biotech. Lett. 7, 255, 1985.

10.10 M. Wayman and R. Parekh, Production of acetone-butanol by extractive fermentation using dibutylphthalate extractant, J. Ferment. Techn. Japan, in press.

10.11 M. Wayman and S.T. Tsuyuki, Fermentation of xylose to ethanol by Candida shehatae, Biotech. Bioeng. Symp. No. 15, 167, 1985.

10.12 M. Wayman and S. Yu, Fermentation os spent sulphite liquor to butanol and ethanol, J. Pulp and Paper Sci. 12 (3) J72, May, 1986.

Publications, continued:

10.13 M. Wayman and S. Parekh, Ethanol and sugar tolerance of Candida shehatae, Biotech. Lett. 7 909, 1985.

10.14 M. Wayman, S. Yu and S. Parekh, Fermentation to ethanol of pentose-containing spent sulphite liquor, Biotech. Bioeng., in press.

10.15 Shiyuan Yu, Ph.D. thesis, University of Toronto, 1986.

10.16 S.R. Parekh, S. Yu and M. Wayman, Adaptation of Candida shehatae and Pichia stipitis to wood hydrolysates for enhanced ethanol production, J. Appl. Microbiol. Biotech. 25, 300, 1986.

10.17 S. Parekh and M. Wayman, Fermentation of cellobiose by pentose-fermenting yeasts, Biotech. Lett. 8, 597, 1986.

10.18 M. Wayman, S. Parekh, E. Chornet and R. Overend, SO2-catalysed prehydrolysis of coniferous wood chips for ethanol production, Biotech. Lett. 8,749, 1986.

10.19 R. Jeng, S. Yu and M. Wayman, Isoenzyme and protein pattern of pentose-fermenting yeasts after adaptation to wood hydrolysates, Appl. Microbiol. Biotech., in press.

10.20 S.R. Sarad and M. Wayman, Performance of a novel continuous dynamic immobilized cell bioreactor for ethanol production, Enzyme and Microb. Tech., in press.

10.21 M. Wayman, C. Seagrave and S.R. Parekh, Ethanol fermentation by Pichia stipitis of combined pentose and hexose sugars from lignocellulose prehydrolysed by SO2 and enzyme saccharified, Process Biochem., in press.

Volume

2

FINAL REPORT
VOLUME II

DEVELOP A NOVEL BIOMASS CATALYSED PRETREATMENT AND HYDROLYSIS FOR COSOLVENT FUEL BUTANOL AND ETHANOL FERMENTATION

RENEWABLE ENERGY DIVISION

PREPARED FOR:

BIOENERGY DEVELOPMENT PROGRAM

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RECENT ADVANCES IN XYLOSE AND CELLOBIOSE FERMENTATION

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Pichia stipitis R has proven itself an excellent pentose fermenter in whole wood and whole grain hydrolysates. Brettanomyces clausenii is an excellent cellobiose fermenter. P. stipitis CBS5776 ferments both pentoses and cellobiose, but not very rapidly. Mixed cultures produce ethanol from partially hydrolysed wood or other lignocellulosics rapidly, efficiently, with high yields of ethanol. The yield of ethanol from SO_2 prehydrolysed aspen in an S.S.F. mode with this mixed culture was 384 L/tonne dry wood, A NEW RECORD. No cellobiase was needed in saccharification.

INTRODUCTION

The direct bioconversion of substrates of cellulosic origin for the manufacture of liquid fuels such as ethanol is a subject of growing interest. One approach to the conversion of cellulosic material into ethanol comprises pretreatment of the lignocellulosics followed by enzymatic or acidic saccharification and fermentation of the sugars so produced to ethanol[1]. Enzymatic saccharification using cellulases of Trichoderma reesei is promising since the process is non-polluting and requires milder conditions for hydrolysis and less expensive equipment. Also total saccharification can be achieved due to the high selectivity of the enzymes[2]. However, conversion times are much longer than for acid hydrolysis; and the cost of enzymes high, thereby requiring recycling of the enzymes for process economy[3]. These cellulases are also deficient in β-glucosidase, resulting in an accumulation of cellobiose during saccharification in addition to glucose. The accumulation of glucose and cellobiose inhibits the activity of the cellulase complex and decreases the rate of cellulose hydrolysis[3]. Supplementation of β-glucosidase from other sources to the T. reesei cellulase complex has been a prerequisite to alleviate the inhibition by preventing excessive cellobiose accumulation[4].

In addition to cellobiose, hydrolysis of biomass yields varying amounts of D-glucose, D-xylose, D-mannose, D-galactose and L-arabinose, depending upon the hydrolysis procedure and the nature of the feedstock[2]. The extent to which these sugars can be fermented to ethanol by yeasts is therefore of paramount importance for commercial exploitation of lignocellulosic biomass to fuel energy.

A few cellobiose-fermenting yeasts have been identified, namely in the Torulopsis, Brettanomyces and Candida species[5,6]. Other investigators have shown that yeasts which can ferment cellobiose to ethanol have a poor capacity for xylose fermentation[7]. Recently, strains of Candida tenuis, Candida shehatae and Pichia stipitis[7] have been identified that ferment cellobiose, xylose and other wood sugars[8,9]. Also, improved strains of P. stipitis and C. shehatae have been reported[10] capable of fermenting pentose-containing lignocellulosic liquors rapidly and efficiently. Such yeasts provide a unique opportunity for integrating the hydrolysis of sugars and their fermentation into a single operation. Direct bioconversion carried out as "Simultaneous Saccharification and Fermentation (SSF)" offers several advantages: The strong inhibition caused by cellobiose and glucose can be circumvented thereby increasing the hydrolysis rate and ethanol yield. The whole process can be carried out in one stage as opposed to 3 or 4 stages for the conventional sequential process (hydrolysis, sugar concentration, product fermentation, and/or microbial protein fermentation). The capital costs of large equipment are much reduced. The presence of ethanol in the fermenter broth reduces the possibility of contamination[11].

Although bakers yeast could successfully be applied in eliminating inhibition by glucose in the SSF process, cellobiose accumulation may still occur and inhibit hydrolysis. Since cellulose hydrolysis is the rate limiting step in the SSF process, means of removing cellobiose is desired other than supplementation of β-glucosidase for process economy.

Incorporating yeasts capable of directly fermenting cellobiose and xylose and other wood sugars directly to ethanol seems an attractive alternative. Such yeasts would allow SSF of the hemicellulose and cellulose fraction, increase the rate of cellulose hydrolysis and possibly reduce the high dose of enzymes and thus eliminate enzyme recycling. P. stipitis CBS 5776 and P. stipitis 'R' offers these merits, however, they are slow cellobiose fermenters. B. clausenii is a potent glucose and cellobiose fermenter but is deficient in fermenting pentose sugars. The objective of the present study is to evaluate the performance of pentose and cellobiose fermenting yeasts in co-cultures for the direct fermentation of wood sugars at rapid and efficient rates; and the possible application of these mixed cultures in the SSF process for lignocellulosics.

We have developed a successful process for producing ethanol utilizing SO_2 prehydrolysed biomass feedstock coupled with enzymatic saccharification and fermentation using P. stipitis, however, the conversion times for the process including hydrolysis and alcoholic fermentations were about 96 hours[1], which is longer than is desirable.

This research is based on the hypothesis that the hydrolytic action of cellulase is very rapid, that the inhibitory effect of cellobiose is also very rapid and that the removal of this inhibitor as soon as it is formed by a cellobiose-fermenting yeast would facilitate the conversion of cellulose to ethanol. The optimum temperature for hydrolysis by cellulase is about 50°C, which is too high a temperature for our best cellobiose-fermenting yeast, Brettanomyces clausenii, which ferments best at about 30°C, and a compromise temperature had to be chosen. We found that at 38°C the rates of hydrolysis and of fermentation were compatible. Also, since we used SO_2-pretreated aspen wood as substrate, there was much xylose present, and this is not fermented by B. clausenii. Pichia stipitis R is an excellent xylose fermenter, but not very efficient in fermenting cellobiose. A mixed culture of this yeast with B. clausenii would achieve the double purpose of removing by fermentation cellobiose and of converting xylose rapidly and efficiently to ethanol. The procedure adopted to test this hypothesis was to add to SO_2-pretreated aspen wood, which had not been washed and still had the hemicellulose sugars in place, cellulase and at the same time a mixed culture of these two yeasts. The results were excellent: in a 48 hour simultaneous saccharification and fermentation, 384 L ethanol/tonne dry weight of aspen were obtained, with 90% of this (345 L/tonne) being obtained in 24 hours. These are the highest yields so far reported of ethanol from aspen wood.

MATERIALS AND METHODS

Micro-Organisms

Brettanomyces clausenii NRRL Y-1414 was obtained from Northern Regional Research Laboratories, U.S.D.A, Peoria, Illinois. It was maintained on YM slant with 2% glucose as the carbon source. Pichia stipitis CBS 5776 was obtained from Centraal Bureau Voor Schimmelcultures (CBS), Yeast Division, Delft, The Netherlands. The R strain of P. stipitis was obtained by adapting the parent strain (CBS 5776) to the wood hydrolysates by semi aerobic cultivation for 24h, centrifuging and recycling the yeast many times. The adapted strain is referred to here as P. stipitis R^{10}. Both Pichia stipitis strains were maintained on YM agar slant with xylose as the carbon source.

Culture media: Du Preez's medium CA containing casamino acids (Difco, Vitamin free), added vitamins and minerals and adjusted to pH 5.0 was used[9]. When added, cellobiose was used at 20 gL^{-1}. The wood sugar mixtures used as substrate contained 20 gL^{-1} D-glucose, 20 gL^{-1} D-xylose, 10 gL^{-1} each of D-cellobiose, D-galactose and D-mannose. The sugars were autoclaved separately from the medium constituents.

Inoculum and fermentation conditions: The inoculum was prepared in 250 mL Erlenmeyer flasks with 100 mL of the medium containing 40 gL^{-1} xylose (in case of P. stipitis strains) and 40 gL^{-1} glucose (in case of B. clausenii), as the carbon source and grown for 48 h at 30°C on a rotary shaker at 150 rpm. Cells were harvested by centrifugation and washed twice with saline, and inoculated in shake flasks at the dry cell concentrations described in the text. The fermentation conditions were the same when evaluating the performance of the parent and the R strains of P. stipitis and the mixed cultures on simulated wood sugar fermentation. All shake flask fermentations having xylose sugar were performed under semi aerobic conditions at 30°C as described earlier[10], and under anaerobic conditions by B. clausenii with cellobiose.

Simultaneous Saccharification and Fermentation (SSF) of SO_2 Prehydrolysed Aspen Wood

Aspen wood chips were prehydrolysed using 3% SO_2 (w/w) as the hydrolysing catalyst at 150°C for 30 mins[12]. The pH of the prehydrolysates containing residual fibre in suspension was raised to 4.8 with $Ca(OH)_2$, the liquid/solid ratio being 10:1. The enzyme system contained cellulase (Meiji Seika), and hemicellulase (Miles) along with vitamin B_{12} and trace elements as promoters[10]. No addition of β-glucosidase was made. The total enzyme added was 15% (w/w) enzyme on fiber. SSF studies with added cellobiose at 20 gL^{-1} were also made. Saccharification without any micro-organism was also performed in parallel to monitor the concentrations of sugars produced on hydrolysis. All SSF experiments were performed in duplicate in a constant temperature water bath at 38° and 125 rpm.

Analysis

Individual wood sugars were determined by HPLC (Waters Sugar Analyzer 1) using an Aminex

HPX-87P column (Bio-Rad) with water as the mobile phase at 65°C, flow rate 0.5 mL/min., using a refractive index detector (Waters 401 RI). Ethanol was determined by GC.

RESULTS AND DISCUSSION

Fermentation of Cellobiose

The ability of P. stipitis 5776 and B. clausenii Y-1414 to produce ethanol from 25 gL^{-1} cellobiose is presented in Table 1. Although both yeast strains ferment cellobiose, B. clausenii was more efficient in fermenting cellobiose, 100% cellobiose fermentation occuring in 20 hours giving a yield of 0.47 g ethanol/g sugar consumed (84% of theory). In case of P. stipitis, some rate limiting step of cellobiose utilization resulted in slower ethanol production. Disaccharides are either split outside or may be taken up intact and hydrolysed inside the cell. Either extracellular hydrolysis or uptake or both could thus be the rate limiting step in cellobiose fermentation by P. stipitis 5776.

Fermentation of a Simulated Wood Sugar Mixture

The five wood sugars namely D-glucose, D-mannose, D-galactose, D-xylose and D-cellobiose were used in a mixture simulating wood hydrolysate in order to determine the utilization pattern that could be expected during SSF of ligno cellulosics. The results are shown in Table 2. Both yeasts initially utilized glucose preferentially and the presence of glucose appeared to retard the fermentation of mannose and galactose more severely in case of B. clausenii than with P. stipitis 5776 (data not shown). Also with P. stipitis, utilization of the other sugars commenced after glucose was depleted and there after simultaneous fermentation of galactose, mannose, xylose and cellobiose occured. The ethanol yield coefficient was similar for both yeasts (0.43 to 0.44 g/g sugar consumed), but the failure of B. clausenii to ferment xylose is reflected by the 29 gL^{-1} ethanol produced by P. stipitis 5776 compared to 21.4 gL^{-1} by B. clausenii under the same conditions.

Using mixed cultures of B. clausenii-and P. stipitis 5776 each at 2.5 gL^{-1} initial cell concentration, almost complete fermentation of simulated wood sugars was achieved with a yield of 0.48 or 92% of theoretical. Ethanol yield was higher in the mixed culture (0.48) compared to 0.42 with P. stipitis or 0.31 with B. clausenii. These results reflect the advantage of co-cultures of B. clausenii and P. stipitis in SSF for successful and rapid fermentation of ligno cellulosics.

Recycling of P. stipitis

Since the economic feasibility of using hydrolysed biomass in an industrial fermentation process depends not only on the successful and rapid fermentation of all the wood sugars but also to the tolerance of these yeasts to toxic substances, adaptation of P. stipitis 5776 to these hydrolysates by repeated recycling was carried out to get strains that would overcome these inhibitions and such limitations[10]. Performance of the recycled R strain and the parent strain on D-xylose and aspen wood hydrolysate are shown in Table 3 and of the glucose: xylose mixture in Figure 1. Fermentation of xylose with P. stipitis R showed improvement in substrate utilization and higher concentration of ethanol. With the parent strain, fermentations were far from complete at the end of 72 hours. Also, fermentation of aspen wood hydrolysates by unadapted cultures were slow and incomplete (Table 3). With P. stipitis R, almost complete fermentation was achieved in 48 hours with ethanol yield close to theoretical[3] (94%). These results were further confirmed with glucose-xylose mixture (Figure 1). The R strain of P. stipitis accumulated almost twice the concentration of ethanol in 72 hours compared to the parent strain. The initial slow rate with the latter culture may be due to the lack of tolerance of high substrate concentration. Improvement in ethanol yields utilizing recycled cells in the fermentation of lignocellulosics by Pachysolen tannophilus, Candida tropicalis and C. shehatae have also been reported.[7,10]

SSF Process

In order to overcome the inhibiting influence of cellobiose on cellulase, wood sugar hydrolysates were fermented simultaneously with saccharification. Neither the optimum temperature for cellulase activity, 50°C, nor for yeast growth, 30°C, were used; instead these SSF runs were made at 38°C, as high as thought feasible for the fermentation. Cellulase and hemicellulase were added and either P. stipitis CBS 5776 or mixed cultures. The results are shown in Table 4 and Figure 2.

Sugar production profiles from SO_2 prehydrolysed aspen wood are shown in Figure 2. Over 80% of the total sugars in aspen wood were hydrolysed in the first 24 hours, with a very slow increase thereafter. This slow rate may be due to the low temperature of saccharification, together with the lack of β-glucosidase. At the end of 48 hours, about 680 kg total sugars/tonne of aspen wood were obtained or about 90% of theoretical yield.

The results of ethanol production using mixed cultures of P. stipitis and B. clausenii in the SSF mode are presented in Table 4. All fermentation were 90% complete in the first 24 hrs and slower thereafter, possibly due to inhibition of cellulase by ethanol. Mixed cultures of P. stipitis R and B. clausenii gave better fermentation rates and a yield of 384 L ethanol/tonne Aspen wood in 48 hrs (85% of theory). Mixed cultures of P. stipitis 5776 and B. clausenii were slightly inferior giving 365 L/tonne. P. stipitis 5776 in pure culture was less efficient resulting in only 73% theoretical ethanol yield. This incomplete fermentation is attributed to the lack of tolerance of this yeast

to inhibitors in the wood hydrolysate. Addition of cellobiose at 20 gl^{-1} or 20% on wood in the SSF process also resulted in high overall ethanol yields and confirmed that if cellobiose accumulated during the course of saccharification in absence of β-glucosidase enzyme it would eventually be rapidly converted to ethanol by these cellobiose-fermenting yeasts. No cellobiose was detected in the fermented hydrolysate at the end of 48 hrs.

The two major cellulose hydrolysate sugars, glucose and cellobiose were each fermented by B. clausenii or P. stipitis. However, the higher ethanol production rate on cellobiose obtained with B. clausenii, which has β-glucosidase activity, is advantageous and the ability to ferment cellobiose rapidly improves hydrolysis and ethanol yields.

P. stipitis 5776 poses the seemingly rare ability to ferment both xylose and cellobiose effectively and compares favorably with other pentose fermenting yeasts used in lignocellulosic fermentation. Some particular problems are sometimes encountered when attempting to ferment all major wood sugars. For example, with C. tropicalis, depletion of glucose and mannose results in consumption of ethanol before the onset of galactose and xylose utilization[13]. Disuxie has also been shown to cause a considerable lag period during the fermentation of sugar mixtures by P. tannophilus and appears to be a common phenomenon during the fermentation of xylose or cellobiose in presence of glucose[14]. However, P. stipitis 5776 does not exhibit such an effect, and fermentation of xylose and cellobiose commences immediately after glucose depletion.

Experiments with mixed cultures of P. stipitis and B. clausenii in simulated wood sugar fermentations produced 12% more ethanol compared to P. stipitis 5776 alone in the same time, suggesting that the use of such mixed cultures would be an advantage in a rapid SSF process.

Low conversion yields and ethanol concentrations found with unadapted fresh cultures were easily overcome by "strain improvement" using the cell recycling technique. How these high ethanol producing yeast strains are generated upon repeated recycling is not known. It may be that recycling results in selection of an adapted strain with derepression of the enzymes required for efficient pentose fermentation[15].

We have reported a process for producing ethanol utilizing SO_2 prehydrolysed biomass feed stock coupled with enzymatic hydrolysis and the use of pentose fermenting yeast[1]. However, the total time for the process was 96 hrs. The present work has shown that yeasts capable of robust cellobiose and pentose fermentation perform faster and better in coupled wood saccharification and fermentation systems. Applying the SSF process to SO_2 prehydrolysed aspen wood with cellulase and hemicellulase but without β-glucosidase and employing mixed cultures of P. stipitis R and B. clausenii not only produced ethanol at a faster rate but also resulted in 5-14% more ethanol compared to P. stipitis 5776 and B. clausenii, and to P. stipitis 5776 alone. Added celliobiose in the SSF process (Table 4) also fermented without difficulty indicating that cellobiose inhibition can be overcome by use of a potent cellobiose fermenter. Also no problems were encountered during the operation at 38°C, thereby indicating the possibility of the application of these yeasts in SSF of lignocellulosics.

CONCLUSIONS

This paper draws attention to the fact that successful SSF processing of lignocellulosics with high pentosans can be achieved rapidly and efficiently without β-glucosidase supplementation when pentose fermenting yeast is coupled with a cellobiose fermenter.

B. clausenii was identified as a potent cellobiose fermenter. All the major sugars that would be released on saccharification of biomass were fermented by P. stipitis 5776. Strain improvement of P. stipitis through recycling represented a useful technique and resulted in rapid fermentation of lignocellulosic hydrolysates. A mixed culture of P. stipitis R and B. clausenii in a single vessel in a SSF mode converted the SO_2 prehydrolysed aspen wood to monomeric wood sugars and the resulting wood sugars to ethanol at a rapid and efficient rate, with yields which are higher than any reported to date. This also shows for the first time the application of coupled pentose and cellobiose fermentation in SSF process, eliminating the need for β-glucosidase supplementation.

Further investigations aimed at eliminating the use of hemicellulase and reducing the amount of cellulase are in progress.

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REFERENCES

1. Wayman, M., Parekh, S., Chornet, E. and Overand, R., Biotechnol. Lett., 8, 749 (1986).
2. Bisaria, V. and Ghose, T.K., Enzyme and Microbiol. Technol., 3, 90 (1981).
3. Howell, J.A. and Stuck, J.D., Biotechnol. Bioeng., 17, 873 (1975).
4. Sternberg, D., Vijaykumar, P. and Reese, M., Can. J. Microbiol., 23, 139 (1976).
5. Freer, S.N. and Detroy, R.W., Biotechnol. Bioeng., 25, 541 (1983).
6. Kilian, S.G., Prior, B.A., Potgieter, J.H. and du Preez, J.C., J. Appl. Microbiol. Biotechnol., 17, 281 (1983).

7. Maleszka, R., Wang, P.F. and Schneider, H., Biotechnol. Lett., 5, 171 (1982).
8. Toivola, A., Yarrow, W., Van der Bosch, E., Van Dijken, J.P. and Scheffers, W.A., Appl. Environ. Microbiol., 47, 1221 (1984).
9. Parekh, S. and Wayman, M., Biotechnol. Lett., 8, 597 (1986).
10. Parekh, S., Yu, S. and Wayman, M., Appl. Microbiol. Biotechnol., 25, 300 (1986).
11. Ghose, T.K., Roy Choudhury and Ghose, P., Biotechnol. Bioeng., 26, 377 (1984).
12. Wayman, M., Tallevi, A. and Winsborrow, B.C., Biomass, 6, 183 (1984).
13. Slapack, G., Edey, E., Mahmourides, G. and Schneider, H., Biotech. Symp. Pulp & Paper Indust., Sept. 12-14, NRCC pub. no. 21205 (1983).
14. Detroy, R.W., Cunningham, R.L. and Herman, A.I., Biotech. Bioeng. Symp., 12, 81 (1982).
15. Jeng, R., Yu, S. and Wayman, M., personal communication.

Table 1. Ethanol production from 25 gL^{-1} cellobiose by *P. stipitis* and *B. clausenii*.

Yeast Strain	Fermentation time (h)	Substrate utilized (%)	Final ethanol concentration (gL^{-1})	Ethanol yield g ethanol/g sugar consumed
P. stipitis CBS 5776	48	100	10.3	0.41
B. clausenii Y-1401	20	100	11.7	0.47

Table 3. Fermentation of D-xylose and aspen wood hydrolysates by adapted *Pichia stipitis*.

Substrate	Strain	Substrate Utilized %	Ethanol gL^{-1}	Yield g ethanol/g sugar utilized
Xylose[a]	*P. stipitis* CBS 5776	73	34	0.44
	P. stipitis R	88	40	0.44
Aspen wood hydrolysate[b]	*P. stipitis* CBS 5776	78	29	0.41
	P. stipitis R	95	41	0.47

a - Initial xylose was 105 gL^{-1}, results reported at 96 hr.

b - Initial total reducing sugars was 92 gL^{-1}, results reported at 48 hr.

Table 2. Ethanol yields from simulated wood hydrolysate by *P. stipitis* and *B. clausenii* and the mixed cultures of both organisms.

Yeast cultures	Cell concentration gL^{-1}	Fermentation time (h)	% substrate utilized	Cellobiose utilized %	Ethanol gL^{-1}	Ethanol yield g/g sugar consumed	Final Yield g ethanol/g total sugar present
B. clausenii	2.5	48	71	100	21.4	0.43	0.31
P. stipitis CBS 5776	2.5	48	95	90	29.0	0.44	0.42
P. stipitis & *B. clausenii*	2.5 + 2.5	48	98	100	33.0	0.48	0.48

* 70 gL^{-1} wood sugars (D-xylose 20 gL^{-1}, D-glucose 20 gL^{-1}, D-mannose 10 gL^{-1}, D-cellobiose 10 gL^{-1}, D-galactose 10 gL^{-1})

Table 4. Ethanol concentrations at various times, %SF with mixed cultures of *P. stipitis* and *B. clausenii* on SO_2 prehydrolysed aspen wood, containing 70 gL^{-1} total reducing sugars.

Yeast culture	Cultural Conditions	Ethanol gL^{-1} T = 2	8	10	24	28	48	% Substrate utilized	Ethanol yield g/g sugar consumed	Final ethanol conversion L/tonne aspen	Ethanol yield on wood % of theory
a) *P. stipitis* CBS 5776	18 gL^{-1} cells[a]	1.6	15	18	23	24	26	85	0.42	328	73.2
b) *P. stipitis* CBS 5776 & *B. clausenii*	18 gL^{-1} cells[a]	1.8	17	20	23	28	29	92	0.44	365	81.5
c) *P. stipitis* R & *B. clausenii*	18 gL^{-1} cells[a]	3.3	19	21	28	29	30	93	0.44	384	85.8
d) *P. stipitis* CBS 5776 & *B. clausenii*	18 gL^{-1} cells[b]	3.0	20	23	29	31	34	98	0.44	441[c]	79.0

a - 5 g SO_2 prehydrolysed aspen wood, 10% dry solids, de Proes salts, 15% enzyme loading (hemicellulase + cellulase).

b - The above conditions plus 20 gL^{-1} cellobiose or 20% on wood.

c - Of which 96 L are are attributable to cellobiose.

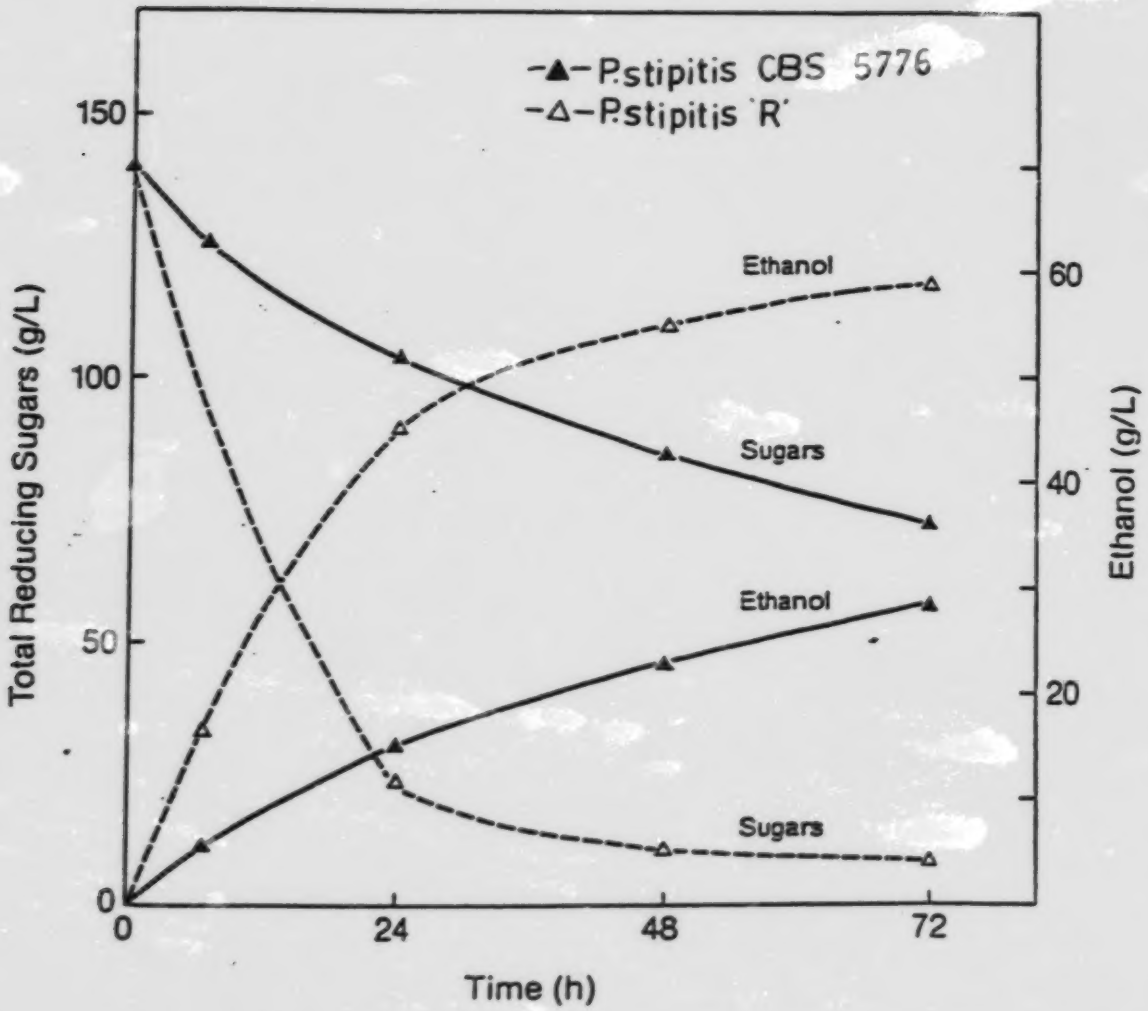

Fig 1

Comparison of ethanol fermentation of glucose/xylose mixtures (70:30) by *P. stipitis* R and *P. stipitis* CBS 5776, cell concentration, 8.5 g dry cell/L

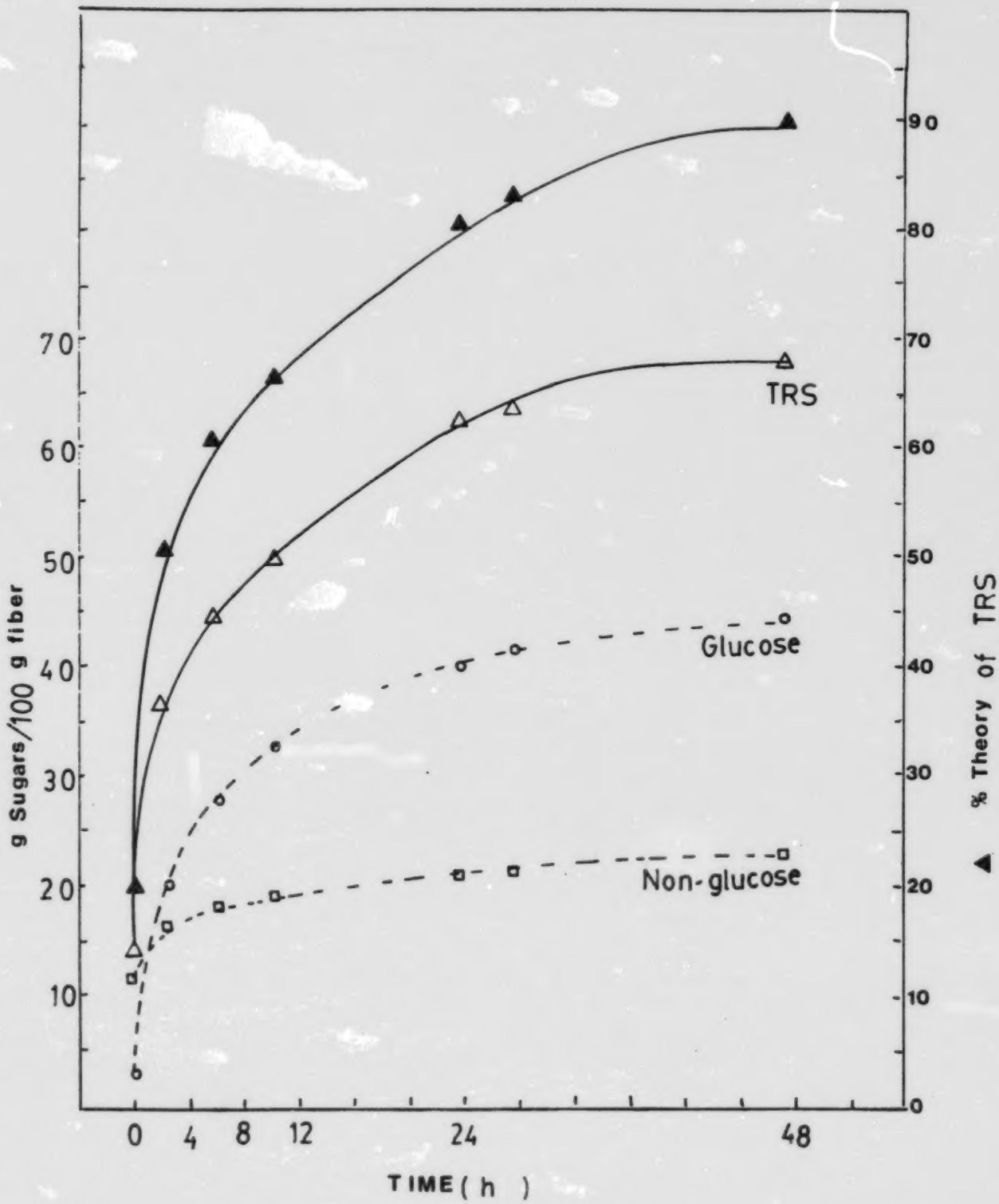

Fig: Time course of saccharification of SO_2 pretreated wood Aspenwood in the presence of cellulase

SO_2 PRETREATMENT FOR HIGH YIELD ETHANOL PRODUCTION

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Pine, aspen and corn stover were prehydrolysed with SO_2 followed by direct enzyme saccharification. The solution of hexoses and pentoses so obtained were fermented by Pichia stipitis R, yielding 372, 346 and 388 L ethanol/tonne. Process and yeast species are novel. A large scale run, 1.5 tonne/h, with coniferous wood at U. de Sherbrooke gave excellent results.

The use of SO_2 as pretreatment or prehydrolysis of lignocellulosic biomass as the first stage of conversion to fermentable sugars, and thence to fuel alcohols, is now well established (1,2). Before forest and agricultural crop residues can be hydrolysed to fermentable sugars, they require pretreatment to render them susceptible to attack by hydrolytic agents. During pretreatment, the hemicellulose of the biomass is hydrolysed, either by steam (autohydrolysis) or by an acid catalyst (prehydrolysis). The fibrous residue, which is a mixture of cellulose and lignin, may then be hydrolysed by acid or enzymes (3). Autohydrolysis (4) is effective in hydrolysing hemicelluloses of deciduous woods such as aspen, and of agricultural crop residues such as corn stover, but has not yet been found useful for coniferous woods such as pine. For these an acid catalyst is required. A comparison of the effectiveness of acid catalysts (5) showed SO_2 to be the most effective for this purpose. This has been studied in the laboratory (2) and on a large scale for coniferous wood (1) with encouraging results. Effective hemicellulose hydrolysis is important because its fermentation increases the yield of ethanol by a considerable margin, ranging from 27% from pine to 65% from corn cobs (6). Successful fermentation of hemicellulose sugars requires yeasts able to ferment pentoses, especially xylose, and when hydrolysis is incomplete, also cellobiose and perhaps other glucose oligomers. We have made very good progress in solving this problem in the laboratory (7) and on an industrial pilot plant scale (8). Among the pentose-fermenting yeasts, Pichia stipitis and Candida shehatae offer advantages over other yeasts because they exhibit efficient ethanol fermentation of xylose, cellobiose (9) and other wood sugars. Improved strains of P. stipitis and C. shehatae are reported (1) having enhanced efficiency of utilization and fermentation of pentose-containing lignocellulosic hydrolysates, and are tolerant of and resistant to toxic substances which may be generated from these feedstocks.

The present paper describes our recent researches in hydrolysis of lignocellulosic feedstocks, using SO_2 prehydrolysis, with some comparison with autohydrolysis in the case of aspen. While we are intensely interested in acid hydrolysis following prehydrolysis in research being carried out in collaboration with St. Lawrence Reactors Limited (11), this paper will confine itself to the results of experiments with enzyme hydrolysis. To compare the status of these two processes in a perhaps oversimplified statement, we could say that the technology of enzyme hydrolysis is well established but the economics remain uncertain, whereas the economics of acid hydrolysis may be more favorable, but the technology requires further research and development, which is proceeding.

METHODS

Raw materials: The feedstocks used in the present investigation were aspen residues from an Ontario wood processing plant, pine from a local sawmill, mixed coniferous woods from Quebec and corn stover from a nearby farm. Their chemical composition is given in Table 1.

Pretreatment: In the laboratory, samples of about 100 g (dry weight) of aspen and corn stover were treated in a 7 L direct steam batch reactor with 3% SO_2 on dry fibre, in the presence of a small amount of water, the liquid/solid ratio being about 2:1. Time and temperature for prehydrolysis were 30 minutes at 75 psig (0.5 MPa),

190°C. In addition several runs were made in the Stake II reactor installed at the Université de Sherbrooke with the collaboration of Professors E. Chornet and R.P. Overend (1). These were large scale, 1500 kg/h, at a residence time of 2 min and pressures ranging from 175 psig (1.2 MPa) to 250 psig (1.7 MPa).

Enzyme saccharification: The pretreated fibres were not washed to remove hemicellulose sugars, as is often done, but were saccharified directly. Enzymatic hydrolysis was carried out in 500 mL shake flasks at 50°C, agitated at 140 rpm for 48 h, assisted by a drop of Triton X100 and stainless steel balls. The pH was adjusted to 4.8 with lime, the liquid/solid ratio being 5:1 to 7:1, i.e., 12 to 16% dry solids. For aspen and coniferous wood hydrolysis, the enzyme system contained cellulase (Meiji Seika), hemicellulase (Miles) and cellobiase (Novo) along with Vitamin B_{12} and trace elements as promoters (1). For corn stover the enzyme mixture also included pectinase (Rohm and Haas) and glucoamylase (Novo) (12). The total hydrolysates containing both hemicellulose and cellulose sugars, were filtered and used as the fermentation substrate.

Fermentation: Pichia stipitis CBS 5776 was adapted to wood hydrolysate by semiaerobic cultivation for 24 h, centrifuging and recycling the yeast many times. The R strain so obtained was maintained on YMP agar with 2% xylose as the carbon source. Inoculum was prepared in YMP medium containing 40 gL^{-1} glucose:xylose 70:30, in 2 L shake flasks on a rotary shaker at 150 rpm and 30°C. Cells were harvested by centrifugation, washed twice with saline and inoculated into fermentation at 14 g cell dry weight/L (freeze-dried weight). Urea and diammonium phosphate were added as N and P sources at 0.1 and 0.2 gL^{-1} respectively. Semiaerobic fermentation was at 30°C for 48-72 h.

Analysis: Wood sugars were determined by HPLC (Waters Sugar Analyzer 1) using Aminex HPX-87P column (BioRad) with water at 65°C as the mobile phase, flow rate 0.5 mL/min, using a refractive index detector (Waters 401 RI). Oligomer content was determined after post-hydrolysis with 4% H_2SO_4 at 120°C for 1 h. Ethanol was determined by GC.

RESULTS AND DISCUSSION

Table 2 compares the results of autohydrolysis and SO_2-prehydrolysis of aspen and of coniferous woods. Autohydrolysis of aspen resulted in about 81% hemicellulose hydrolysis, and about 65% of the resulting sugars were monomeric. With the addition of a small amount of SO_2 to the steam, hemicellulose hydrolysis was over 91%, and 94% of the resulting sugars were monomeric. When the same procedures were applied to coniferous wood, only 60% of the hemicellulose sugars were hydrolysed by steam, and of these only 60% were monomeric, whereas when SO_2 was added to the steam, 89% of the hemicellulose sugars were freed, and of these, 98% were monomeric. Thus while autohydrolysis may be employed for pretreatment of aspen and the results may be considered acceptable, pretreatment of coniferous wood with steam alone gave poor yields of hemicellulose sugars. In all cases, the addition of a small amount of SO_2 improved prehydrolysis to near theoretical levels.

Table 3 gives the results of SO_2-prehydrolysis of corn stover. While hemicellulose hydrolysis was not complete under the conditions employed, which may not be optimal for this feedstock, the yield of hemicellulose sugars was 79% and of these 94% were monomers. High glucose is probably due to the presence of starch in corn stover.

Table 4 presents the results of fermentation of the enzymatically saccharified fibre following autohydrolysis or SO_2-prehydrolysis. The superiority of SO_2-pretreatment for the coniferous wood is clearly demonstrated, more than twice as much ethanol being obtained compared to autohydrolysis. With aspen as feedstock, the difference in ethanol production of not so great, only about 8% more ethanol being obtained after SO_2-prehydrolysis then after autohydrolysis. We do not have a similar comparison for corn stover.

Yields of ethanol from the SO_2-prehydrolysed, enzyme saccharified feedstocks varied from 73.6 to 81.0%, all based on the maximun yields which could be expected from their chemical composition. It is, of course, clear that in such a 3 stage reaction, prehydrolysis, hydrolysis and fermentation, 90% yields at each stage would result in only 73% overall. Perhaps this is what we are experiencing.

CONCLUSIONS

SO_2 prehydrolysis renders coniferous woods, aspen and corn stover readily amenable to enzymatic saccharification. The resulting mixture of pentose and hexose sugars are fermentable by R yeasts to ethanol in good yields: indeed these yields may be the highest yet reported for these feedstocks. This process, which combines flexibility of feedstock with robust hydrolysis and fermentation would lend itself to large scale industrial application if the economics of enzyme saccharification could be brought into an acceptable range (13).

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REFERENCES

1. Wayman, M., Parekh, S., Chornet, E., and Overend, R., Biotechnol. Lettrs., 8, 749 (1986).
2. Wayman, M., Tallevi, A., and Winsborrow, B., Biomass, 6, 183 (1984).
3. Wayman, M., Lora, J.H., and Gulbinas, G., ACS Symposium Series No. 90, pp. 183-201 (1978).
4. Lora, J.H. and Wayman, M., TAPPI, 61(6), 47-50 (1978).
5. Wayman, M., in Cellulose: structure, modification and hydrolysis, eds. R.A. Young, R.M. Rowell, Wiley, New York, 1986, pp. 265-279.
6. Wayman, M., Biomass, 1987, in press.
7. Wayman, M. and Tsuyuki, S.T., Biotechn. and Bioeng. Symp. No. 15, 167-177 (1985); Wayman, M., Yu, S. and Parekh, S.R., Biotechn. and Bioeng., in press.
8. Yu, S., Personal communication: report on operation of pilot plant December 22, 1986.
9. Parekh, S.R. and Wayman, M., Biotechnol. Lettrs., 8, 597 (1986).
10. Parekh, S.R., Yu, S. and Wayman, M., Appl. Microbiol. Biotechnol. (1986) (in press); Jeng, R., Yu, S. and Wayman, M., Can. J. Microbiol., in press.
11. Arsarsson, P.G., Holysh, M., Tallevi, A., Wayman, M. and Parekh, S.R., Proceedings, Chemical Engineering Annual Conference, Sarnia, Ontario, October 1986.
12. Wayman, M. and Parekh, S., Biotechnol. Lettrs., 7, 909 (1985).
13. Pourquie, J. and Glikmans, G., Proceedings, VII International Symposium on Alcohol Fuels, October 1986, Paris, France.

Table 1 - Summative Analysis of Softwood, Hardwood and Corn Stover

	% of dry weight		
	coniferous[a] wood	aspen	corn stover
Total polysaccharides	70.0	68.0	68.3
Klason lignin	26.0	20.0	15.2
Acid soluble lignin	0.6	1.33	1.0
Extractives	4.4	2.86	9.5
Ash	-	-	2.0
Other	-	7.81	4.0
Total	100.0	100.0	100.0
Polysaccharides			
Glucose	48	43	53.3
Non-glucose	22	25	15.0

[a]mixed pine and spruce chips from Quebec

Table 2 - Prehydrolysis of Aspen and Coniferous Woods

Pretreatment Conditions	Glucose (%)	Total Sugars (%)	Fiber Residue
Aspen:			
Autohydrolysis (steam) 1.72 MPa (250 psig), 2 min			
Monomer	2.35	16.15	72.06
Oligomer	0.85	9.25	
Total	3.20	25.40	
% of theory			
Glucose	6.8		
Non-glucose	81.0		
Prehydrolysis, 3% SO_2 on wood, 75 psig, 30 min			
Monomer	2.89	27.08	69.9
Oligomer	0.60	1.61	
Total	3.49	28.69	
% of theory			
Glucose	7.4		
Non-glucose	91.6		
Coniferous Wood:			
Autohydrolysis (steam) 1.72 MPa (250 psig), 2 min			
Monomer	2.24	11.33	75.6
Oligomer	1.93	7.54	
Total	4.17	18.87	
% of theory			
Glucose	7.8		
Non-glucose	60.0		
Prehydrolysis, 2% SO_2 on wood, at 1.72 MPa (250 psig), 2 min			
Monomer	5.41	26.56	71.3
Oligomer	0.14	0.54	
Total	5.55	27.10	
% of theory			
Glucose	10.5		
Non-glucose	89.4		

Table 3 - Prehydrolysis of Corn Stover

Pretreatment Conditions	Glucose (%)	Total Sugars (%)	Fiber Residue
Prehydrolysis, 3% SO_2 on fiber, 75 psig (160°C)			
Monomer	7.82	20.67	71.14
Oligomer	0.93	1.08	
Total	8.75	21.75	
% of theory			
Glucose	15.0		
Non-glucose	79		

Table 4 - Fermentation to Ethanol of Combined Hemicellulose and Cellulose Hydrolysates

Pretreatment conditions	Sugar yield after enzymatic hydrolysis		Yields after fermentation					
	total sugars (gl^{-1})	% on raw material	reducing sugars (gl^{-1})	ethanol (gl^{-1})	g ethanol/ g sugar consumed	% ethanol on fiber	l ethanol/ dry tonne of raw material	ethan % of theor
a) coniferous wood								
SO_2-prehydrolysed fiber	134	71.7	6	55	0.43	29	372	76.8
steam autohydrolysed fiber	36	32	3	15.1	0.45	13	166	34.7
b) aspen								
SO_2-prehydrolysed fiber	123	68	5.6	52	0.44	27	346	73.6
steam autohydrolysed fiber	72	61	6	30	0.45	25	320	66.9
c) corn stover								
SO_2-prehydrolysed fiber	120	65	6	52	0.44	30.2	388	81.0

A NEW CONTINUOUS DYNAMIC IMMOBILIZED CELL FERMENTER

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Yeast cells, immobilized on rotating disks in a horizontal cylindrical continuous bioreactor, ferment sugar solutions of 100 to 200 g/L rapidly and efficiently, residence times for glucose, sucrose and cane molasses being 1.6, 1.6 and 3.6 hours, sugar utilization 90%, with ordinary bakers' yeast. Pichia stipitis R in this bioreactor ferments spent sulphite liquor containing both pentoses and hexoses efficiently and rapidly.

INTRODUCTION

Recently there has been worldwide interest in the production of ethanol by fermentation as an alternative to chemical feed stock and as a gasoline extender. Most of the ethanol fermentation practised today is by batch methods. The main drawbacks of batch fermentations are start up and shut down times, high labour cost, large fermenter volume requirements and low productivity. Improving productivity of the fermenter requires the development of a "high rate" process with reduced fermenter and operating costs, which essentially means a) continuous operations, b) high cell density in the bioreactor, c) minimized residence time, d) distillation feed containing a high concentration of ethanol, and e) rapid and continuous removal of inhibitory end products, especially CO_2 gas. Several new technologies have been developed and proposed to meet the above goals including vacuum fermenters[1], cell recycling[2], hollow fiber membrane reactors with recycling[3] and immobilization of whole cells in polymeric gels[4-6]. The use of vacuum-assisted fermentation with recycling involves the high cost of centrifuges, while the use of ultrafiltration membranes, apart from being costly, have many mechanical problems. The use of viable whole cells immobilized in solid gel matrices such as beads has gained wide spread attention and has been studied in packed bed[7] and fluidized-bed reactors[8]. However, due to the diffusional resistance of substrate or product and the requirement of rapid removal of CO_2 from the reactor, together with microbial viability for long term production of ethanol, these systems have relatively low efficiency and find limited applications.

Clyde[9] developed a rotating fiber disc fermenter suitable for carrying out a 3-phase reaction involving liquid substrate, solid immobilized cell biocatalyst and CO_2 gas. In his study, bacterial cells, Zymomonas mobilis, were trapped in porous fiber discs by physical adsorption and rotated in a bioreactor containing fermentable substrate. Fiber discs offer a large surface area for immobilization and the rotation results in better mixing between the 3-phases and rapid removal of evolved CO_2 from the liquid phase. However this reactor has serious shortcomings: a) the amount of cells loaded by simple adsorption in the fiber discs is low, and b) the rotation of the fiber discs results in the detachment of cells from the surface of the fiber discs and consequently substantial loss of microorganisms from the reactor. The end results are high investment costs for centrifuges to separate cells from the distillation stream and decreased efficiency of the reactor during long periods of operation. Also, no literature data are available on the performance of this system with immobilized yeasts, the microbes utilized today in the fermentation ethanol industry. These considerations prompted us to modify the reactor system and study the use of rotating fiber discs with immobilized yeasts.

To maintain high cell density in the reactor and to overcome the negative effect of cell loss we have introduced the technique of lightly immobilizing the yeast cells adsorbed in the fiber discs with low concentrations of calcium alginate gel. By such a procedure yeast cells are trapped, immobilized and bound to the supports, forming a thin film on the surface of the fiber discs. Such a method maintains high cell density without causing excessive cell leakage when the discs are rotated, and the thin

film of gel coated cells offers least mass transfer resistance through the porous fiber discs. The present paper describes the performance of this modified continuous dynamic immobilized cell (CDIC) bioreactor with respect to ethanol production from sugars normally used in industrial practice and highlights the merits of the CDIC bioreactor for potential industrial application.

MATERIALS AND METHODS

Organisms: Saccharomyces cerevisiae was a dried bakers' yeast (Fleischman's) obtained locallly. Pichia stipitis R was a strain derived from Pichia stipitis CBS 5776 recycled several times in wood hydrolysate[10]. This strain was used in the present study for comparison with S. cerevisiae since it has the ability to rapidly ferment pentose sugars derived from lignocellulosic feed stock[11].

Medium: The feed medium consisted of reducing sugars at concentrations ranging from 45 to 200 gL^{-1} from various substrates, supplemented with 1 gL^{-1} yeast extract, 1 gL^{-1} NH_4Cl and 0.1 gL^{-1} urea at pH 5.0. Cane molasses was obtained from Canada West Indies Molasses Co., Mississauga, Ontario, and acid liquified corn wet milling waste (12% dry solids) from a nearby (30,000 bushels/day) corn wet milling plant. Cane molasses was diluted to the required sugar strengths with tap water, pH adjusted to 5 and steam sterilized. Corn wet milling waste water was enzymatically saccharified at pH 4.8 with 0.1% (w/v) glucoamylase (Novo) for 6 h at 50°C.

Bioreactor: A schematic diagram of the experimental apparatus is shown in Figure 1. The reactor was a stainless steel cylinder, 35 cm long and 11 cm in diameter, in which fiber discs (9.5 cm in diameter) were mounted on a central stainless steel shaft (0.5 cm in diameter), spaced at 5 discs/cm. The discs are made of a woven, rigid porous cellulose acetate fabric, cut to uniform circles, then randomly perforated to increase the flow of medium through the fermenter. They were rotated at 40 rev min^{-1} by a constant speed motor connected to the shaft. The upper transparent removable cover had orifices for feed inlet and overflow, and 3 chimneys for CO_2 outlet. The outlet gas lines were connected to 3 condensers which returned moisture and volatile components to the fermenter. Chilled water (4°C) was circulated through the condensers. The bioreactor temperature was controlled by placing it in a constant temperature incubator chamber. The bioreactor was placed slightly inclined to the horizontal, the outlet being above the inlet.

Cell immobilization: Cells of P. stipitis R for immobilization were grown in 4 L Erlenmeyer flasks with 2 L of medium of the following composition (gL^{-1}): D-glucose 40, yeast extract 3, malt extract 3, peptone 5 at pH 5.0 on a rotating shaker at 150 rpm. The cells were harvested in the late exponential phase of growth and concentrated by centrifugation at 1000 rpm (164 g) for 10 min. The CDIC bioreactor with the fiber discs in place was sterilized with 5% formaldehyde solution and washed several times with hot sterile distilled water. Sodium alginate (Fisher Scientific) was used as the cell entrapment material. Concentrated free cells were suspended in 0.85% NaCl to which 0.1% sodium alginate was added and mixed thoroughly to form a thick uniform suspension. The resulting slurry (at 30°C) was introduced into the previously sterilized bioreactor and the fiber discs were rotated by the motor. During this period, the alginate coated yeast cells were physically entrapped in and on the fiber discs. After 4 hrs, a 2% $CaCl_2$ solution was added, causing the cells to be bound in the form of a thin film of calcium alginate on the surface of the discs. The film was allowed to harden in $CaCl_2$ solution for 6 h. Untrapped cells were leached out by washing the reactor with sterile saline, until the effluent was clear.

Bioreactor operating procedure: The liquid phase volume in the fermenter, 2.0 L, was 60% of the total bioreactor volume of 3.4 L. The cells immobilized on the fiber discs were rotated constantly at 40 rev min^{-1}. A positive displacement peristaltic pump was used to vary the liquid feed flow rate. The flow rate of the medium was determined by timing the volume collected at the overflow outlet of the bioreactor, and the dilution rate of the reactor was calculated from the reciprocal of the liquid residence time in the fermenter[12]. The pH was not controlled during the experiment because pH varied only slightly from the initial value of 5 to a final value of 4.8. This slight change in pH (in the short residence time) was considered to have no significant effect on the fermentation rate of the yeasts. Continuous operations were conducted at different volumetric flow rates and at each flow rate the system was considered to have reached steady state once 5 fermenter volumes had passed, and when ethanol, reducing sugars and free cell concentrations remained constant as evidenced by 3 successive samples. Only steady state data are reported.

Analysis: Ethanol was determined by GC using n-propanol as the internal standard[13]. D-glucose was measured with a glucose analyzer (YSI - Yellow Springs Instruments). Total reducing sugars were analysed by the Nelson-Somogyi method[14]. Yeast cell dry weight immobilized per unit reactor volume (gL^{-1}) was measured optically at 660 nm, using a Spectronic 20 (Bausch and Lomb), and converted to gL^{-1} with the aid of a calibration curve[15].

RESULTS

Table 1 presents the main results of fermenting the different substrates. Since most of the ethanol producing companies practise fermentations leading to 85% or greater substrate conversions[16,17], only those results are reported here in which over 95% substrate utilization occurred. Lower substrate fermentation was obtained when flow rates were too great. From

the data the least mean residence time for 85% or more substrate fermentation, and ethanol productivity at that flow rate, were calculated. At shorter residence times, conversion was less.

Table 1 shows S. cerevisiae to ferment sucrose and glucose in less than 2 hours with 88 and 90% substrate utilization respectively. The yield was 0.50 g ethanol/g sugar consumed with these two substrates at 55 and 54 gL^{-1}. Ethanol concentration in the effluent was 24 gL^{-1}, or above 3% by volume. Under the same conditions, cane molasses with a sugar concentration of 195 gL^{-1} was completely fermented in 3.6 hours with high productivity, ethanol concentration being 10.6% by volume.

Fermentation of corn wet milling waste having 115 gL^{-1} fermentable sugars was complete in 2.5 h with yields close to theoretical and productivity of 22.4 $gL^{-1}h^{-1}$.

Under the same conditions Pichia stipitis R was able to ferment sucrose, glucose and cane molasses to 86 to 92% substrate utilization and to produce good yields of ethanol based on sugar consumed. However the retention times were longer in each case than experienced with S. cerevisiae, being 2 h for sucrose and glucose and 5 h for cane molasses.

The yeast P. stipitis R was chosen because it ferments pentoses readily and efficiently. Samples of spent sulphite liquor from a Quebec pulpmill containing 43 gL^{-1} total sugars, of which 28% or 12 gL^{-1}, were xylose, was fermented with this yeast, with the results shown in Table 1. The spent liquor was relatively slow to ferment, 14 h being required for 91% substrate utilization. Considering the high proportion of the sugars which are xylose, the conversion efficiency at 0.45 was quite good. By steam stripping the spent sulphite liquor to reduce toxicity[15] utilization of sugars was improved and fermentation time was reduced to 10 hours. Fermentation of xylose was complete in the steam-stripped liquor.

Operational stability: In order to determine the operational stability of the CDIC fermenter, it was operated continuously at a fixed flow rate of 0.55 Lh^{-1} (dilution rate = 0.27 h^{-1}, 3.6 h mean residence time) with S. cerevisiae loaded at 56 gL^{-1} cell density (based on total reactor volume) for a period of 15 days on a cane molasses medium having about 195 gL^{-1} sugars. Figure 2 shows the plots of ethanol, total reducing sugars, free cell concentration and pH of the effluent as functions of operating time. The data show that during the first 10 days, the values of ethanol and sugars in the effluent remained almost constant with no change in the steady state, but beyond 10 days there was a steady decrease in ethanol concentration with corresponding increase in unutilized substrate. The effluent biomass concentration increased from 0.22 gL^{-1} to 0.57 gL^{-1} dry weight cells over the 15 day period. There was not much change in pH. From the data presented in Figure 2, various kinetic parameters such as volumetric ethanol productivity, percent feed sugars utilized, and ethanol yields were calculated and plotted as functions of time in Figure 3. The value of volumetric ethanol productivity declined from 23 $gL^{-1}h^{-1}$ and 9[illegible] substrate utilization to 20 $gL^{-1}h^{-1}$ at 83% substrate utilization in 15 days. At this time the ethanol yield fell from 0.48 to 0.45 g ethanol/g sugar consumed. No contamination was noticed during the continuous fermentation operation and only a small amount of sludge from molasses adhered to the discs, the rest leaving the reactor in the effluent.

DISCUSSION

Ethanol productivity of about 23 $gL^{-1}h^{-1}$ from cane molasses and corn wet milling waste water in the CDIC fermenter was higher and its performance better than those reported for continuous culture[18], tower fermenters[19], a rotating packed drum reactor[6] and immobilized cells[20]. It also compares favourably with pilot plants tested in Japan with calcium alginate immobilized beads[21] and polyethylene glycol photo crossed-linked resins[22] as carrier. Although ethanol productivities as high as 100-120 gL^{-1} have been reported with fermenters coupled with an external ultrafiltration unit[2] or with membrane reactors[3], scale up of such technology with industrial substrates such as molasses, cane juice, hydrolysed starch and lignocellulosic materials containing high solids which would clog the membranes and would need special sanitation care, may well be impractical.

While the spent sulphite liquor was relatively slow to ferment compared to the other substrates, sugar conversion to ethanol at 0.45 represents over 90% of theoretical, and xylose fermentation was complete. Using this yeast in place of the conventional S. cerevisiae could be expected to result in about 20% more ethanol. In addition, the liquor would be more completely desugared, resulting in a purer lignin for other conversion operations such as vanillin manufacture. The increase in yield due to the use of this yeast would be much greater for hardwood spent sulphite liquor and for hardwood hydrolysates, which are high in xylose.

The initial activity of the CDIC bioreactor decreased by about 8% during 15 days of continuous operation. On the basis of these data, the estimated "half life" of the reactor operated at 0.55 Lh^{-1} (dilution rate = 0.27 h^{-1}) was 98 days. Since the reactor activity remained constant for the initial 10 days and declined slowly thereafter, to maintain continuous prolonged activity of the reactor, the cells in the reactor need reviving every 10 days. To achieve this, O_2, growth factors, sterols and unsaturated fatty acids may be added to the feed substrate. This results in maintaining continuous growth and viability with prolonged catalytic activity of yeast cells in immobilized bioreactors[21].

Table 3 shows the advantages of using the CDIC reactor for ethanol production compared to conventional batch fermentations. The CDIC fermenter has productivity of alcohol 12-18 times higher than conventional batch fermentation and enables the fermentation to be completed in a few hours compared to 2-3 days required in batch processes. The process also represents lower initial investment and operating costs. The newly designed CDIC reactor described shows great potential for industrial application, since it is able to handle any sugar solutions presently used industrially and possibly any which may be obtained from biomass resources in future. It also offers the following benefits:

- high cell loading and maintaining this at high levels
- sterilizable and reuseable supports
- both immobilization and fermentation are carried out in the same vessel, with low chance of contamination. This also decreases the initial investment costs
- easy elimination of CO_2
- high ethanol output
- maintenance of high viability of yeast cells
- minimal product inhibition due to low retention time.

CONCLUSION

The newly designed CDIC fermenter shows great promise of fermenting sugar-containing substrates rapidly and efficiently. It has industrial potential and could be developed for economical production of ethanol on a continuous basis.

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REFERENCES

1. Cysewski, G.R. and Wilke, C.R. Biotechnol. Bioeng. 1977, 19, 1125-1143
2. Rogers, P.L., Lee, K.J. and Tribe, D.E. Process Biochem. 1980, 15, 7-11
3. Cheryn, M. and Mahaia, M.A. Process Biochem. 1984, 19, 204-208
4. Cheetham, P.S.J., Blunt, K.W. and Bucke, C. Biotechnol. Bioeng. 1979, 21, 2155-2168
5. Chang, H.N., Joo, I.S. and Ghim, Y.S. Biotechnol. Lett. 1984, 6, 487-492
6. Lee, C.W. and Chang, H.N. Enzyme Microbiol. Technol. 1985, 7, 561-563
7. William, D. and Munnecke, D.M. Biotechnol. Bioeng. 1981, 23, 1813-1825
8. Nagashima, M., Azuma, M., Noguchi, S., Inuzuka, K. and Samejima, H. Biotechnol. Bioeng. 1984, 26, 992-997
9. Robert Clyde, U.S. Patent, No. 4,407,954 1983.
10. Parekh, S.R., Yu, S. and Wayman, M. Appl. Microbiol. Biotechnol. 1986, 25, 300-304
11. Wayman, M., Parekh, S., Chornet, E. and Overend, R.P. Biotechnol. Lett. 1986, 8, 749-752
12. Krouwel, P.G. and Kossen, N.W.F. Biotechnol. Bioeng. 1981, 23, 651
13. Wayman, M. and Tsuyuki, S.T. Biotechnol. Bioeng. 1985, Symp. 15, 167-177
14. Somogyi, M. J. Biol. Chem. 1952, 195, 19-23
15. Yu, S., Wayman, M. and Parekh, S. Biotechnol. Bioeng. 1986, in press
16. Jakson, E.A. Process Biochem. 1976, 11, 29-30
17. Kolot, F.B. Process Biochem. 1980, 15, 2-8
18. Ghose, T.K., Tyagki, R.D. Biotechnol. Bioeng. 1979, 21, 1387
19. Netto, C.B., Destruchaut, A. and Goma, G. Biotechnol. Lett. 1985, 7, 355-360
20. Ghose, T.K. and Bandhopadhyay, K.K. Biotechnol. Bioeng. 1980, 22, 149
21. Technical report on "Continuous Alcohol Fermentation with Immobilized Growing Yeast", 1983, Kyowa Hakko Kogyo, Co. Ltd., Japan
22. Technical report on "Continuous Alcoholic Fermentation by Immobilized Yeast Cells", 1983, J.G.C. Corp., Japan

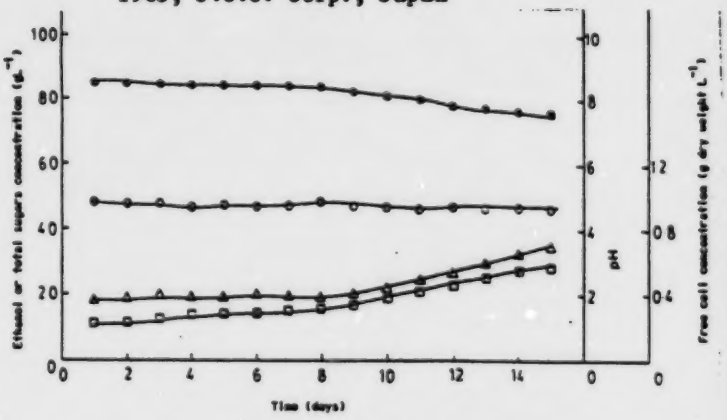

Figure 2. Performance of CDIC fermenter with 19.5% molasses sugar as a function of time at constant feed flow rate of 0.55 Lh^{-1} (D = 0.27 h^{-1}); ( ) ethanol; ( ) total reducing sugars; ( ) pH; ( ) free cell concentration.

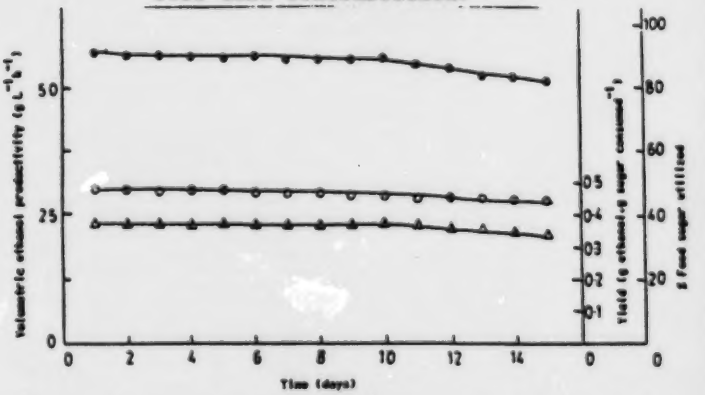

Figure 3. Stability of CDIC bioreactor with 19.5% molasses sugar fermentation as a function of time at constant feed flow rate, 0.55 Lh^{-1}, ( ) % sugar utilized; ( ) ethanol yield; ( ) volumetric ethanol productivity.

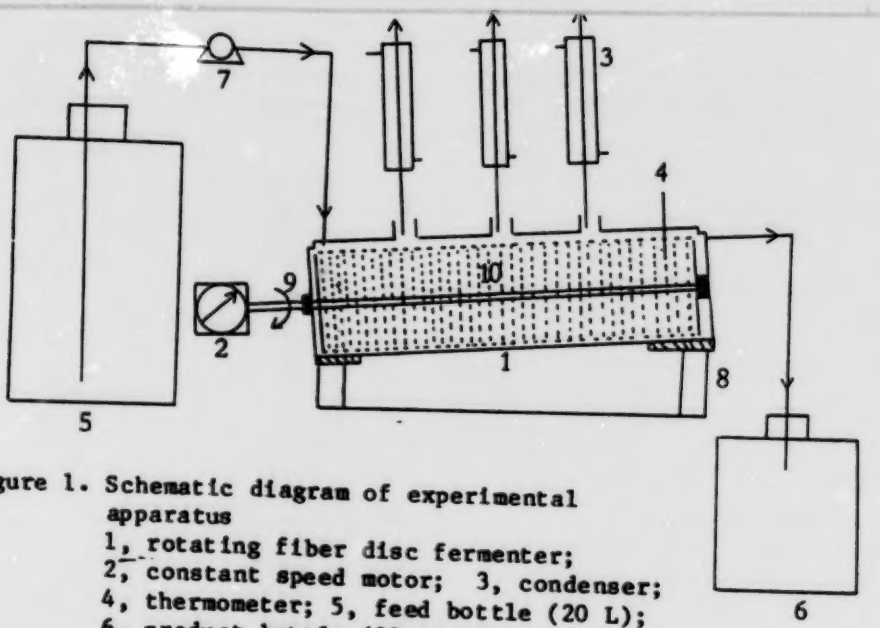

Figure 1. Schematic diagram of experimental apparatus
1, rotating fiber disc fermenter; 2, constant speed motor; 3, condenser; 4, thermometer; 5, feed bottle (20 L); 6, product bottle (20 L); 7, peristaltic pump; 8, stand; 9, central shaft; 10, fiber discs. Volume of medium 2.0 L; total volume 3.4 L.

Table 1. Performance of continuous dynamic immobilised cell fermentor

Substrate	Sugar concentration (gL^{-1})	Sugar utilisation (%)	Ethanol in effluent (gL^{-1})	Ethanol productivity ($gL^{-1}h^{-1}$)	Retention time (h)	Yield g ethanol/ g sugar consumed	Cell loading in reactor (gL^{-1})	Cells in effluent (gL^{-1})
			Saccharomyces cerevisiae					
Sucrose	55	88	24.2	15.2	1.6	0.50	56	0.18
Glucose	54	90	24.3	15.2	1.6	0.50	56	0.21
Cane molasses	195	90	83.0	23.0	3.6	0.48	56	0.22
Corn wet milling waste	115	98	56.0	22.4	2.5	0.50	56	0.22
			Pichia stipitis R					
Sucrose	54	86	22.8	11.4	2.0	0.49	56	0.18
Glucose	50	92	22.0	11.0	2.0	0.48	56	0.21
Cane molasses	190	86	78.0	15.6	5.0	0.48	56	0.22
Softwood spent sulphite liquor	43	91	18.3	1.3	14.0	0.45*	49	0.10
Softwood s.s.l. steam stripped	42	97	17.9	1.8	10.0	0.45**	49	0.11

* - 95% of xylose consumed
** - 100% of xylose consumed

Table 2. Features of continuous alcoholic fermentation in a CDIC bioreactor by *S. cerevisiae*

Process and features	Conventional process	Hills-Blanet process[22]	Immobilised yeast cells in CDIC reactor
Fermentation Type	Batch	Batch	Continuous
Cell concentration (gL^{-1})	2-3	9.6	56
Final ethanol concentration (gL^{-1})	100-110	55-80	85
Yields from sugars (%)	85-86	85-86	90
Ethanol productivity ($gL^{-1}h^{-1}$)	1.3-2.0	2-4	23
Fermentation time (h)	70-76	15-25	2-4

10-4

OFFPRINTS FROM CELLULOSE: STRUCTURE, MODIFICATION, AND HYDROLYSIS
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15

Comparative Effectiveness of Various Acids for Hydrolysis of Cellulosics

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The acid hydrolysis of cellulose and of cellulosics such as wood for the production of fermentable sugars has long been studied in the laboratory. Industrial facilities for wood hydrolysis have been operating for the past 40 years. Even earlier, under the stimulus of wartime conditions, European and North American facilities were built for acid hydrolysis of wood. The sugars produced were fermented to ethanol, which is an acceptable motor fuel. With the rapid expansion of the petrochemical industry in the years following about 1935, most such fermentation facilities became uneconomical, and most ethanol production switched to petroleum-derived feedstock. Some fermentation ethanol continued to be produced with molasses as the substrate. The rapid increase in the price of oil in the past 10 years has altered that position substantially, and now once again large amounts of ethanol are made by fermentation. This reversion to fermentation as the preferred process has created a need for a cheap and widely available source of fermentable sugars. Cellulosics such as wood and agricultural crop residues appear to meet these requirements.

Brazil's numerous fermentation fuel alcohol plants are fed by the by-products of their large cane sugar industry. Their experience demonstrates that fermentation ethanol is a perfectly satisfactory motor fuel at 95% alcohol 5% water. At least 500,000 Brazilian automobiles operate on undried alcohol continuously, and most of the rest of their fleet operate on this fuel some of the time—for example, on weekends, when only alcohol is available at the gas station. Brazil

also sells a "gasohol" with 20% anhydrous ethanol in gasoline, and this is considered a transition until their fermentation capacity can be increased to meet the full demand.

In the United States there were, at the end of 1982, 65 fermentation alcohol plants in operation, mostly based on corn. Of these, half made 4×10^6 L/yr or more, and five each made 200×10^6 L/yr or more. The economics of ethanol production is highly sensitive to scale (1), and these very large plants make ethanol by fermentation more cheaply than it can be made from petrochemicals. The total output of these U.S. plants is about 2×10^9 L/yr—a large volume but even so, less than 1% of U.S. motor fuel demand.

All of these fermentation plants are based on grain or sugar, which are human food or animal feed. There is widespread intuitive concern about driving automobiles on food- or feed-based fuel. This conflict is exacerbated by the need for relatively good land for these crops, resulting in competition for land. Inevitably, it is feared, such competition will raise food costs, with harmful social consequences. As a result, much attention has been devoted to the much more difficult problem of converting cellulosics—wood and agricultural crop residues—to power alcohols (2).

In Canada, waste wood and waste crop residual—surplus to needs of soils and animals—are in relatively concentrated locations so that they can be delivered to a conversion facility at acceptable costs (3, 4). There is enough to make 6×10^9 L/yr of fermentation ethanol—that is, about 20% of current Canadian gasoline consumption. In addition, there is available a great deal of abandoned farmland and other poor land on which to plant fast-growing wood hybrids and other energy crops, enough to supply all of the rest of the motor fuel Canada requires. It would require only 5×10^6 ha of relatively poor land to provide the full amount of cellulosic raw material for the full amount of motor fuel.

In the United States, logging and wood processing residue are estimated (5) at 53×10^6 tonnes/yr, having a potential ethanol yield of 7×10^9 L/yr. To supply the rest of the motor fuel for the United States, by planting fast-growing wood hybrids and other energy crops, would require $20–25 \times 10^6$ ha of relatively poor land. These calculations suggest that the provision of motor fuel in North America entirely from replenishable resources is technically feasible.

Of the many factors that delay implementation of such a fuel supply program, failure to agree on a satisfactory technology is significant. All commercial plants for producing fermentable sugars from wood have been based on hydrolysis by dilute sulfuric acid in percolation towers. There are about 40 such plants in operation in the Soviet Union. Such processes have poor yields, many operating problems (6), and unsolved metallurgy, and they give rise to humic substances which inhibit fermentation (7). Furthermore, the economics of such plants is questionable (8).

A hopeful alternative appeared with the introduction of autohydrolysis as a pretreatment for cellulose (9). Autohydrolysis—that is, steam cracking followed by rapid quenching—is highly effective for hydrolysis of hemicelluloses of de-

ciduous woods such as poplar and of agricultural crop residues. In addition, much of the lignin becomes soluble in dilute alkali, or in organic solvents, and extraction of the lignin leaves a relatively pure cellulose for hydrolysis or for other uses. The autohydrolysis–extraction–hydrolysis process (10) results in good yields of fermentable sugars and ethanol (11). It is schematized in Figure 15.1.

Until now this process has not proved useful for coniferous woods such as pine or spruce, which are the most plentiful residues of the forest industries. In an effort to improve the process by extending its raw material base and to find a satisfactory alternative to sulfuric acid as hydrolysis catalyst, we have studied catalyzed autohydrolysis, which should more properly be called prehydrolysis, and have compared several acids as hydrolysis catalysts for production of fermentable sugars.

BACKGROUND

Cellulosics pose special problems as a feedstock for hydrolysis because of their physical and chemical properties. Wood and agricultural crop residues are bulky solids, which for reasonably rapid and uniform chemical treatment require subdivision into relatively fine particles. Physical methods of subdivision such as grinding are expensive, expecially for wood. One of the virtues of the "steam explosion" processes is that wood chips can be converted to finely divided fibrous products suitable for further processing as fluid slurries while at the same time effecting useful chemical changes. Chemically, cellulose is homogeneous, but biomass has several components, namely, cellulose, hemicellulose, lignin, and extractives. Hemicelluloses, like cellulose, are polymeric carbohydrates (12), but cellulose is a highly crystalline polymer of glucose exclusively, and hemicelluloses are amorphous polymers made up of many monomers including D-glucose, D-xylose, D-mannose, L-arabinose and D-galactose. In addition, hemicelluloses also contain acetyl and uronic acid groups.

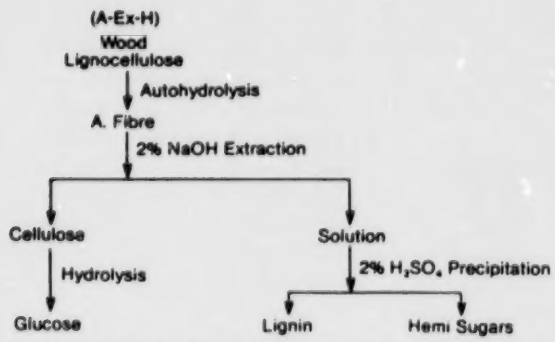

Figure 15.1 Autohydrolysis-extraction process.

The proportions of these sugar residues vary widely in the hemicelluloses of different plant species, with coniferous woods having a higher proportion of glucomannans and deciduous woods more xylans and arabinoxylans. This is an important distinction, since mannose is readily fermentable by ordinary yeasts, whereas the pentoses, xylose and arabinose, are fermentable by only a few unusual yeasts. The carbohydrate composition of hemicelluloses of straws, bagasse, and other agricultural crop residues is more complex, but in general they tend to be very high in pentosans.

In plants, the hemicelluloses are combined with lignin although by relatively weak bonds. Lignin is an amorphous phenolic polymer made up of three monomers—coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol (13). The proportions of these monomeric residues vary with species. The lignin of conifers is richer in residues derived from coniferyl alcohol, whereas that from deciduous species has relatively more sinapyl-derived residues. In consequence of this difference, the lignin in conifers is more condensed and less tractable to chemical attack than the lignin in hardwoods. Grasses and agricultural crop residues have relatively little lignin, and they are relatively high in *p*-coumaryl derivatives.

Extractives may vary in quantity from 1 to 7%—a few species have more—and these are highly species-specific. Generally, they include fats and fatty acids, resin acids such as abietic acid, sterols ("phytosterols"), and a great number of other aliphatic and aromatic organic chemicals. These may affect processing conditions (14).

In addition to these compounds, pectins may be present in crop residues, and, finally, all cellulosics contain inorganic ash, which may be less than 0.5% in wood but may be a few percent of silica in some crop residues.

Thus cellulosics are highly heterogeneous both physically and chemically. The rates of reaction of the various components with hydrolyzing agents are quite different. In particular, hemicelluloses are hydrolyzed by dilute acids at a much more rapid rate and at much lower temperature than cellulose. The use of high temperature for dilute acid hydrolysis of cellulose has resulted in decomposition of the sugars formed in hydrolysis, and the resultant poor yields have led to a continuing search for better processes for hydrolysis of cellulosics.

Among the first cellulose hydrolyzing agents are the three strong acids—72% H_2SO_4, 41% HCl, and 85% H_3PO_4. The action of all three acids is to dissolve the carbohydrates completely at low temperatures (4–20°C). With biomass, the residue is mainly lignin (Klason lignin in the case of H_2SO_4; Willstatter lignin with HCl). The dissolved carbohydrates are mainly oligomers which may be hydrolyzed almost completely to monomers by careful dilution to 3–6% acid and heating at 100–120°C for 30–360 min. These fundamental observations have been used as a basis for industrial installations, using fortified HCl and concentrated H_2SO_4. The strong acid processes that reached an industrial level have been described by Wenzl (15). A more recent survey by Kosaric et al. (5) is based on Ladisch (16) and brings the literature on strong acid hydrolysis to about 1979.

Since then, interest in HCl hydrolysis has continued, particularly as a result

of a project undertaken by Battelle-Geneva (17). In the United States, Goldstein at North Carolina State University and Gaddy at the University of Arkansas have continued studies of HCl (18). In addition, considerable research effort has been directed to HF (19). Of these various processes, those based on 41% HCl have industrial potential, but the cost of recovery of the rather expensive acid is a deterrent to large scale implementation (see also Chap. 17).

All of today's industrial installations and many pilot plants use dilute sulfuric acid as the hydrolysis catalyst. The existing industrial facilities are nearly all in the Soviet Union, with one in Brazil and another in New Zealand, both in the start-up phase. Russian technology has been described by Tokarev (20). The hydrolysis reactors in all of these plants are percolation towers in which batches of wood chips are exposed to dilute (about 0.5–1%) H_2SO_4 at high temperature (to about 190°C) for a long time (2–4 hr). Sugar solutions are withdrawn from the bottom of the towers.

The Brazilian plant employs the latest Russian titanium-based metallurgy and is generally based on their technology but modified to produce both fermentation alcohol and metallurgical coke. The New Zealand technology is a version of the New Zealand Forest Research Institute pilot plant (6, 7), probably modified along the lines suggested by Wayman (8). The Brazilian plant will use eucalyptus as the feedstock, and the New Zealand plant will use pine sawmill residues. Of the several pilot plants in operation, those at New York University (21) and at the Toronto pilot plant of Biohol use extruders as the reactors. The latter has a capacity of about 1 ton of wood feed per day, including processed crop residues. The sugars produced are fermented to alcohol by *Zymomonas mobilis* (22). The extruders have very high capacity considering their small size and have not experienced the serious metallurgical problems that have characterized the percolation tower installations. However, they have not been operated continuously over a long enough time for a definitive statement on corrosion. The other problems associated with dilute sulfuric acid, namely low yields (about 50% of theory) and the formation of fermentation inhibitors, remain.

Grethlein (23) has proposed a different approach to dilute H_2SO_4 hydrolysis of celiulosics by the use of a continuous plug flow reactor at very high temperatures, up to 280°C, for very short times. Such a reactor would take advantage of the kinetics described by Saeman (24), who demonstrated that the rate of cellulose hydrolysis increases faster with increasing temperature than does the rate of glucose destruction in the acid medium. At the high temperature used by Grethlein, yields of 70–80% were obtained. A pilot plant scale reactor capable of operation at high temperatures for short times is under construction (25).

Because of the heterogeneous nature of cellulosic biomass, laboratory and pilot plant studies have generally adopted a two-stage approach to hydrolysis (5, 10, 16). The first stage, prehydrolysis or autohydrolysis depending on conditions, is designed to hydrolyze hemicelluloses, with the resulting sugars removed before the second hydrolysis stage (cf. Chap. 16). In the industrial percolation towers a similar effect is achieved by beginning the hydrolysis reaction at a relatively lower temperature, 150°C, and gradually raising the temperature

to 190°C. The sugars are removed as formed, from the bottom of the reactor. We have generally adopted a two-stage approach. Because of the limitations of our equipment, we have generally carried out the hydrolysis in several steps. The sugars are removed as formed to give good yields (11).

To overcome the problems encountered in industrial practice of dilute acid hydrolysis of wood, paticularly low yields, corrosion, and the formation of fermentation inhibitors, we have compared several acids as prehydrolysis and hydrolysis catalysts. Another object of our work has been to broaden the range of feedstocks that can be treated by autohydrolysis, including conifers.

AUTOHYDROLYSIS OR PREHYDROLYSIS

The raw materials used in this research were red pine residues and aspen chips. Their analyses are given in Table 15.1. Samples of these woods were heated in small autoclaves in the presence of a small amount of water, with a liquid/solid ratio of 4:1 for autohydrolysis and with very dilute SO_2 solutions for prehydrolysis. The time at temperature for autohydrolysis was 15 min at 190°C and for prehydrolysis was 20 min at 150°C. The results are shown for pine in Table 15.2 and for aspen in Table 15.3.

In pine autohydrolysis, about 59% of the hemicellulose nonglucose sugars were hydrolyzed to soluble carbohydrates, and of these about 22% occurred as oligomers rather than monomers. In the presence of a small amount of SO_2, hemicellulose was hydrolyzed completely with a somewhat smaller amount, 17%, as soluble oligomers. In this prehydrolysis, the fibrous residue was less than on autohydrolysis, 69% compared to 74%. The alkali-soluble lignin decreased from about 9% to about 5%.

Aspen hemicelluloses are much more readily hydrolyzable than those of pine under conditions of autohydrolysis, producing 88.4% of the theoretical yield of nonglucose sugars. In the presence of SO_2 this was raised to the full theoretical level. There is a large difference in the completeness of hemicellulose hydrolysis.

TABLE 15.1 Summative Analysis

	% of Dry Weight	
	Red Pine Sawmill Residues	Aspen Poplar Chips
Extractives	5.0	5.4
Polysaccharides	68.0	67.7
(of which glucan)	(50.7)	(42.2)
Lignin:		
Klason	25.8	21.5
Acid-soluble	1.2	5.4
Total	100.0	100.0

TABLE 15.2 Autohydrolysis or Prehydrolysis of Pine

	Glucose %	TRS %	Fiber Residue	Alkali Extract
	Autohydrolysis (Steam), 190°C, 15 min			
Monomer	1.52	11.78	74.33	9.22
Oligomer	2.32	3.32		
Total	3.84	15.10		
% of Theory				
Glucose	6.9			
Nonglucose	59.2			
	Prehydrolysis, 2% SO_2/wood, 150°C, 20 min			
Monomer	5.29	20.55	69.06	4.91
Oligomer	—	4.32		
Total	5.29	24.87		
% of Theory				
Glucose	9.5			
Nonglucose	102.9			

In autohydrolysis, 41.7% of the soluble sugars are oligomers, whereas in the presence of SO_2 catalyst, only 5.3% are oligomers. While the fiber residue is similar in the two procedures (69.1% and 67.1%), the alkali-soluble lignin is much greater in autohydrolysis than in prehydrolysis—14.4% compared to 7.9%. The reduced solubility of the lignin is due to acid-catalyzed polymerization (26).

One can conclude that the use of a small amount of SO_2 as catalyst in

TABLE 15.3 Autohydrolysis or Prehydrolysis of Aspen

	Glucose %	TRS %	Fiber Residue	Alkali Extract
	Autohydrolysis (Steam), 190°C, 15 min			
Monomer	1.41	15.36	69.1	14.40
Oligomer	1.47	11.00		
Total	2.88	26.36		
% of Theory				
Glucose	6.2			
Nonglucose	88.4			
	Prehydrolysis, 3% SO_2/wood, 150°C, 20 min			
Monomer	2.56	29.07	67.1	7.86
Oligomer	0.81	1.62		
Total	3.37	30.69		
% of Theory				
Glucose	7.8			
Nonglucose	102.0			

pretreatment of these woods permits the use of lower temperatures and results in complete hydrolysis of the hemicellulose. The solubility of the lignin in alkali is decreased in the presence of SO_2 under these conditions, presumably because of acid-catalyzed polymerization.

HYDROLYSIS OF PINE WOOD

The above results led us to adopt the general procedure of a first-stage acid-catalyzed prehydrolysis, followed by an alkaline extraction and a second-stage acid hydrolysis, with the same acid used in both prehydrolysis and hydrolysis.

The results of SO_2 acid-catalyzed hydrolysis are reported in Table 15.4. Very high yields were obtained, 84.2% of theory for glucose and 92.2% of theory for total reducing sugars, using 2% SO_2 on fiber with a liquid/solid ratio of 4. The conditions were 0.5% SO_2 solution, at 150°C for 20 min at temperature in prehydrolysis and 1% SO_2 on residual fiber for a total time-temperature equivalent of 24 min at 190°C for hydrolysis. The residue, mainly lignin, was 34.1% of the starting material. The higher total reducing sugar yield is, of course, a consequence of complete hydrolysis of hemicellulose in prehydrolysis. Of the total sugars, 12% occurred as soluble dimers and oligomers, and the remaining 88% was monomeric sugars. It is believed that these are the highest yields reported for dilute acid hydrolysis of cellulose.

In Table 15.5 are given the corresponding results of sulfuric acid hydrolysis. The yields are also good, 86.5% of the glucose in pine as sugars, although almost one-third of these are dimers and oligomers (cf. 11). The yield of total reducing sugars, 83.1%, is high, although significantly lower than that obtained with SO_2, 92.2%. The ligneous residue was almost the same, 34.3% compared to 34.1%.

TABLE 15.4 SO_2 Hydrolysis of Pine Sawmill Residues

	Glucose			Total Reducing Sugars		
	Monomer		Oligomer	Monomer		Oligomer
Prehydrolysis	5.53		1.37	25.28		4.56
Hydrolysis	34.60		5.50	35.31		3.85
	40.13	+	6.87	60.59	+	8.41
Total		=	47.00		=	69.00
% of Theory	71.9	+	12.3	81.0	+	11.2
Total		=	84.2		=	92.2

Prehydrolysis: 2.00% SO_2 on fiber: liquid/fiber = 4, SO_2 solution = 0.5%; 150°C, 20 min at temperature.

Hydrolysis: 1.0% SO_2 on residual fiber, 190°C, 4 × 4 min at temperature, total time–temperature equivalent 24 min at 190°C.

Ligneous residue: 34.09%.

TABLE 15.5 H_2SO_4 Hydrolysis of Pine Sawmill Residues

	Glucose			Total Reducing Sugars		
	Monomer		Oligomer	Monomer		Oligomer
Prehydrolysis	4.11		3.38	23.74		1.75
Hydrolysis	29.19		11.56	36.08		0.58
	33.30	+	14.94	59.82	+	2.33
Total		=	48.24		=	62.15
% of Theory	59.7	+	26.8	81.0	+	3.1
Total		=	86.5		=	83.1

Prehydrolysis: 3.00% SO_2 on fiber: liquid/fiber = 4, SO_2 solution = 0.75%; 150°C, 20 min at temperature.

Hydrolysis: 1.5% SO_2 on residual fiber, 190°C, 4 × 4 min at temperature, total time–temperature equivalent 24 min at 190°C.

Ligneous residue: 34.33%.

In Table 15.6 the results of hydrolysis with these and other acids—nitric, hydrochloric, and formic—are presented. It is clear that SO_2 is the most effective catalyst. The results with HCl and formic acid were quite disappointing, since HCl has been proved to be a highly effective hydrolysis catalyst for starch (27) and the autohydrolysis effect has been attributed to formic acid. Formic and acetic acids are split from the hemicellulose in the wood during steaming.

While different optimal concentrations are used in the experiments reported in Table 15.6, the ratio of $[H^+]$ to carbohydrate is remarkably constant. In one series of experiments, an attempt was made to augment the effectiveness of SO_2 by adding just enough formaldehyde to form the addition compound, which has a higher dissociation constant than H_2SO_3. However, the effects observed were the same as those found for SO_2 alone.

There are important benefits of SO_2 as a hydrolysis catalyst, particularly as

TABLE 15.6 Hydrolysis of Pine Sawmill Residue: Comparison of Acids

	% on Fiber		Yield: % of Theory			
			Glucose		Total Reducing Sugars	
	Prehydrolysis	Hydrolysis	Monomer	Total	Monomer	Total
SO_2	2	1	71.9	84.2	81.0	92.2
H_2SO_4	3	1.5	59.7	86.5	80.0	83.1
HNO_3	2	1	53.2	72.4	68.8	77.4
HCl	1.3	0.65	47.0	57.6	70.5	66.7
Formic	3	—	0.8	7.0	6.5	14.8

compared to sulfuric acid, which has been the industrial standard to date. Since SO_2 is a gas and can be added either ahead of or with the steam, it distributes better through the wood substance than H_2SO_4, to give a more uniform reaction. Some of the difficulties with H_2SO_4 are attributable to uneven distribution in the wood. In addition, SO_2 costs less than H_2SO_4, less is used, and the cost of neutralization following hydrolysis is reduced.

There is much experience in the pulp and paper industry in generation and use of SO_2 in pressure vessels. Therefore we can expect that stainless steels are available that are resistant to attack by SO_2, although metallurgy associated with H_2SO_4 at these temperatures remains a serious problem. While it is apparent that the lignin is somewhat polymerized during prehydrolysis, thereby reducing the solubility in dilute NaOH, it does not display the humic characteristics of H_2SO_4-treated lignin. Lignosulfonates made by the reaction of SO_2 with lignin are the basis of a substantial chemical industry such as vanillin production. Preliminary work suggests that these lignins will be quite suitable for such chemical conversion. The amout of SO_2 used, a total of 3% on fiber, is much less than the amount used in sulfite pulping of wood, which is about ten times as much.

The use of SO_2 as a hydrolysis catalyst for cellulosics has attracted attention for quite some time. Although there is little on this subject in the ordinary technical literature, the patent literature has many examples, one of the earliest is by Ewen and Tomlinson (28). These men built a commercial plant in Georgetown, North Carolina, during World War I. Saeman (29) comments that this plant, and another like it at Fullerton, Louisiana,

> produced alcohol profitable into the 20's. Both plants benefited from very low cost sawdust and a lack of environmental controls. The operators coped successfully with corrosion problems presented by dilute *sulphuric acid* at over 170°C, and with fermentation of impure and variable substrates. The hydrolysis was carried out by adding a small amount of acid to green wood and heating with steam. Essentially these same conditions could be used today in a batchwise or continuous configuration for prehydrolysis or for pretreatment of lignocellulose.

Saeman's statement about sulfuric acid raises some questions, since the Ewen and Tomlinson patent described only the use of gaseous SO_2, "an amount of SO_2, say, equal to about three percent by weight of the (dry weight of the) sawdust." Later Tomlinson patents (30) mention dilute sulfuric acid or sulfurous acids. There is no suggestion in these patents that sulfuric acid is superior to sulfurous acid or SO_2 gas.

TWO-STAGE SO_2 HYDROLYSIS OF OTHER CELLULOSICS

The procedure adopted for hydrolysis of cellulosics was SO_2 prehydrolysis for 20 min at 150°C in 0.5 or 0.75% SO_2 solution (2–3% on fiber). The hemicellulose

sugars were then washed from the fibrous residue with water, and the fibrous residue was hydrolyzed with 1% SO_2 on fiber (0.25% solution) at 190°C, using the procedure described in reference 11.

The results are shown in Table 15.7. As expected, hemicellulose hydrolysis was essentially complete in all cases. However, the amount of sugar recovered from the residual cellulose varied widely among these five species of biomass. The procedure gave good results for pine, 84.3% of the theoretical yield of glucose. All other glucose yields were less than 70%, varying from 68.7% for aspen to only 54.5% for wheat staw. Since in all cases the amount of fibrous residue at the end of the process was about what would be expected from the lignin content, the difference is due not to differences in rate of hydrolysis but rather to differences in the rates of destruction of the glucose as formed; or perhaps a better formulation might be in our failure to preserve the glucose as formed.

FUEL ALCOHOL YIELD FROM CELLULOSICS

Based on the yields of hexose and pentose sugars, yields of ethanol and butanol–acetone–ethanol have been estimated and are listed in Table 15.8. Ethanol yields are based on fermentation with *Candida Shehatae* (31).

Fermentation by *Clostridium acetobutylicum* to butanol–acetone–ethanol (32) would make use of both hexose and pentose sugars. Corn cobs, wheat straw, and pine hydrolyzates would give the highest yields of these fuels; yields from hydrolyzates of poplar and bagasse would be about 20–29% less.

TABLE 15.7 Sugar Yields on Two-Stage SO_2 Hydrolysis of Cellulosics[a]

	Red Pine Sawmill Residue	Aspen Poplar Chips	Bagasse Louisiana	Corn Cobs	Wheat Straw
Prehydrolysis					
Glucose	6.9	3.3	3.0	3.0	2.5
Nonglucose sugars	21.1	23.3	24.8	42.9	43.8
Hydrolysis					
Glucose	40.1	28.9	20.4	20.5	20.2
Nonglucose sugars	(1.0)	3.3	4.0	7.1	2.0
Total					
Glucose	47.0	32.2	23.4	23.5	22.7
Nonglucose sugars	20.1	26.6	28.8	50.0	45.8
% of Theoretical Yield					
Glucose	84.3	68.7	60.4	66.2	54.5
Nonglucose	103.7	91.5	101.0	99.0	113.2

[a] Percentage of dry raw material weight.

TABLE 15.8 Fuel Alcohol Yields on Two-Stage SO_2 Hydrolysis of Cellulosics[a]

	Red Pine Sawmill Residue	Aspen Chips	Bagasse Louisiana	Corn Cobs	Wheat Straw
Ethanol	403	342	297	406	379
Butanol–acetone–ethanol	252	220	196	276	257

[a] Estimated from sugar yields; L/tonne dry raw material.

EQUIPMENT CONFIGURATIONS

Some equipment previously discussed (10) as suitable for autohydrolysis would, if made of 316 ELC stainless steel or better, prove suitable for SO_2 hydrolysis. The Masonite gun configuration shown in Figure 15.2 and the Stake reactor in Figure 15.3 are capable of accepting wood chips and processing them through SO_2 prehydrolysis. Since prehydrolysis is about 150°C, saturated steam pressure at this temperature is about 4 atm (380 kPa), and the commercial equipment of these two designs can easily withstand this or higher pressure. Alternatives may include defibrators and extruders (21). Extraction of lignin following prehydrolysis can be carried out with conventional washers, diffusers, or presses. It is an optional step, which would only be taken if markets for the lignin are available.

There are several configurations of equipment suitable for SO_2 hydrolysis. A continuous plug flow reactor has proved very successful in starch hydrolysis and can be modified for cellulose hydrolysis. A schematic of the St. Lawrence reactor is shown in Figure 15.4, and a model of a full-scale reactor, now in commercial operation on starch, is shown in Figure 15.5. Modifications of the

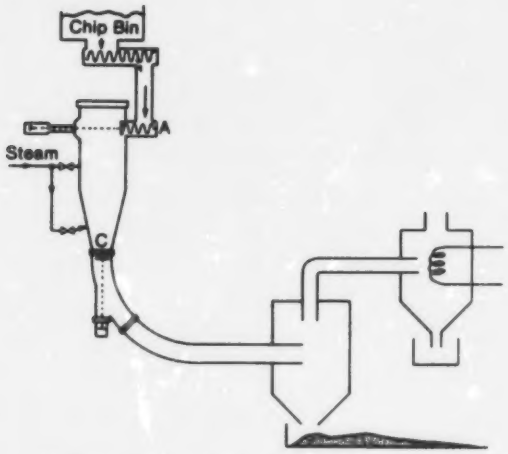

Figure 15.2 Masonite gun configuration.

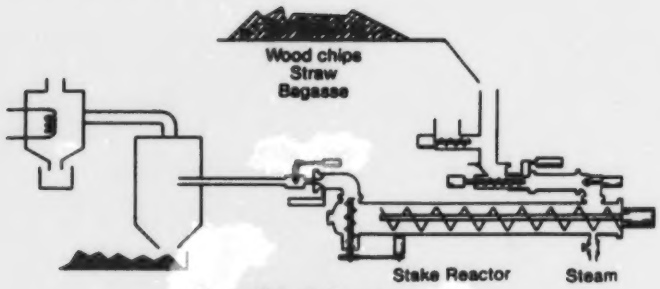

Figure 15.3 Stake reactor.

St. Lawrence reactor for cellulose hydrolysis will include provision for operation at higher temperature and possibly for removal of sugar as formed.

Existing commercial cellulose hydrolysis plants are based on dilute sulfuric acid percolating through a bed of wood. These would profit by conversion from H_2SO_4 to SO_2 catalyst, especially in the modified configuration suggested in references (1) and (8). This would lower costs, protect lignin, and substantially reduce problems of metallurgy.

SUMMARY AND CONCLUSIONS

Of several acids tested as catalysts in hydrolysis of pine sawmill residues, SO_2 proved to give the highest yield of total reducing sugars—92% of theoretical. In the same tests, the yield with a sulfuric acid catalyst was 83%. These yields were obtained in a three-stage process, prehydrolysis, followed by alkaline extraction of lignin followed by hydrolysis. For some purposes, the alkaline extraction is optional.

Prehydrolysis with SO_2 has advantages over autohydrolysis (steam cracking). In the absence of SO_2, pine hemicellulose was resistant to hydrolysis, with only 59% hydrolyzed to sugars, whereas in the presence of SO_2, hemicellulose hydrolysis was complete. Aspen hemicellulose is much more readily hydrolyzed by steam; 88% yields of hemicellulose sugars are realized. However, in the

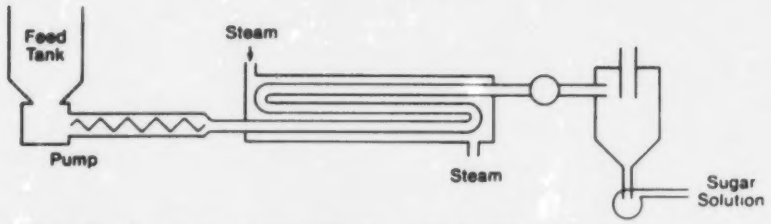

Figure 15.4 Plug flow reactor schematic. (Courtesy of National Organizing Committee, Symp. on Alcohol Fuels, Ottawa).

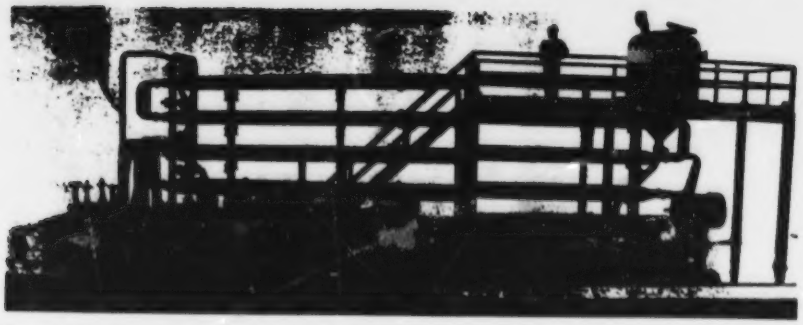

Figure 15.5 Photograph of plug flow reactor model which is now in full-scale operation. (Courtesy of National Organizing Committee, Symp. on Alcohol Fuels, Ottawa).

presence of SO_2 the hemicellulose was completely hydrolyzed. Furthermore, much lower temperatures could be used with SO_2 catalyst—150°C compared to 190°C with steam alone. Also, much more of the hemicellulose sugars occurred as monomers with SO_2 catalyst, about 95%, whereas in autohydrolysis only 58% of the hemicellulose sugars were monomeric.

Compared to the more usual H_2SO_4 catalyst, SO_2 as a gas distributes better in the wood; it is of lower cost and less is required, thereby lowering the cost of neutralization before fermentation. Stainless steels that fail with H_2SO_4 are resistant to SO_2. Lignin quality is better preserved.

Well-proven equipment is available for the various stages of this process. A continuous plug flow reactor can be used for hydrolysis, and the present St. Lawrence starch reactor is undergoing modification for this use.

REFERENCES

1. M. Wayman and A. Dzenis, *Can. J. Chem. Eng*, 62, 699, (1984).
2. Proceedings, International Symposium on Ethanol from Biomass, Royal Society of Canada, Winnipeg, Oct. 1982.
3. R. D. Hayes, "Availability of Crop Cellulosics for Ethanol Production," in ref. 2, 1982, pp. 118–141.
4. M. R. Clarke and C. B. R. Sastry, "Availability of Wood Cellulosics," in ref. 2, 1982, pp. 142–160.
5. N. Kosaric, A. Wieczorek, G. P. Cosentino, R. J. Magee, and J. E. Prenosil, in *Biotechnology*, H. J. Rehm and G. Reed, eds., Vol. 3, Verlag Chemie, Weinheim, 1983.
6. R. J. Burton, "The New Zealand Wood Hydrolysis Process," in ref. 2, 1982, pp. 247–270.
7. K. Mackie, K. Deverell, and I. Callander, "Aspects of Wood Hydrolysis via the Dilute Sulphuric Acid Process", in ref. 2, 1982, pp. 271–311.
8. *Assessment of the New Zealand Forest Research Institute Process for Pine Wood Hydrolysis and Fermentation to Ethanol*, Dept of Energy, Mines and Resources of Canada, Ottawa, 1983.
9. J. H. Lora and M. Wayman, *Tappi*, 61 (6), 47–50 (1978); M. Wayman and J. H. Lora, *Can.*

Pat. 1,147,105; K. Okamura and H. Morikawa, in *Recent Technologies and Prospects of Wood Chemicals*, J. Nakano and T. Higuchi, eds., CMC Report No. 40, CMC Publishers, Kyoto, Japan, 1983.

10. M. Wayman, "Alcohol from Cellulosics: The Autohydrolysis–Extraction Process," *Proceedings IV International Alcohol Fuels Technology Symposium*, Guaruja, Brazil, Vol. I, 1980, pp. 79–89.
11. M. Wayman, J. H. Lora and E. Gulbinas, "*Chemistry for Energy*," ACS Symp. Ser. No. 90, American Chemical Society, Washington, D.C., 1978, pp. 183–201.
12. S. A. Rydholm, *Pulping Processes*, Interscience, New York, 1965.
13. K. Freudenberg, in *Lignin Structure and Reactions*, J. Marton, ed., Advances in Chemistry Series No. 59, American Chemical Society, Washington, D.C., 1966.
14. W. E. Hillis, *Wood Extractives*, Academic, New York, 1962.
15. H. F. J. Wenzl, *The Chemical Technology of Wood*, Academic, New York, (1970).
16. M. R. Ladisch, *Process Biochem.*, 14(1), 21, (1979).
17. J. P. Sachetto and A. Johansson, ' The Battelle–Geneva Fractionation Process," presented at the 186th ACS National Meeting, Washington, Aug. 28, 1983.
18. T. J. Laughlin, D. R. Coleman, M. V. Kilgore, C. L. Lishawa, and M. H. Eley, "The Production of Fuel-Grade Ethanol from the Cellulose in Municipal Solid Waste," presented at the Solar and Biomass Energy Workshop, Atlanta, GA, April 17–19, 1984.
19. M. C. Hawley, Chapter 17 in this volume; C. M. Ostrovski, H. E. Duckworth, and J. C. Aitken, "Canertech's Ethanol-from-Cellulose Program," *Proc. VI Int. Symp. Alcohol Fuels Technol.*, II, 187–191 (1984).
20. B. I. Tokarev, in *The Chemistry of Wood*, N. I. Nikitin, ed., English translation, 1966.
21. B. Rugg, Report SERI/TR-1-9386-1, 105 pp., 1982.
22. G. R. Lawford, B. H. Lavers, D. Good, R. Charley, J. Fein, and H. G. Lawford, "Zymomonas Ethanol Fermentation: Biochemistry and Bioengineering," in ref. 2, 1982, pp. 482–507.
23. H. E. Grethlein and A. O. Converse, "Continuous Acid Hydrolysis for Glucose and Xylose Production", in ref. 2, 1982, pp. 312–336; D. R. Thompson and H. E. Grethlein, *Ind. Eng. Chem. Prod. Res. Dev.*, 18(3), 166–169 (1979).
24. J. F. Saeman, *Ind. Eng. Chem.*, 37, 43–52 (1945).
25. Anonymous, "Renewable Fuels Report," Canadian Renewable Fuels Association, Ottawa, May 1, 1984, p. 5.
26. M. Wayman and M. G. S. Chua, *Can. J. Chem.*, 57, 2603–2611 (1979).
27. C. B. Keim, J. Nagasuye, and P. G. Assarsson, "Preparation of Acid Hydrolysed Starch Substrates and Their Fermentability," XI Int. Carbohydr. Symp., Vancouver, Abstract V-8, 1982.
28. M. F. Ewen and G. H. Tomlinson, *U.S. Pat.* 763,473, June 28, 1904.
29. J. F. Saeman, "Acid Hydrolysis of Cellulosics," in ref. 2, 1982, pp 231–246.
30. G. H. Tomlinson, *U.S. Pats.* 1,032,448 and 1,032,450, July 16, 1912.
31. M. Wayman and S. T. Tsuyuki, "Fermentation of Xylose to Ethanol by *Candida shehatae*", *Biotech. and Bioeng.*, in press; J. C. du Preez and J. P. van der Walt, *Biotech. Lett.* 5, 357–362 (1983).
32. M. Wayman, G. R. Husted, J. D. Santangelo, and S. Levy, "Butanol Production by Extractive Fermentation Using Fluorocarbons," Proc. VI Int. Symp. Alcohol Fuels Technol, Vol. II, 1984, pp. 234–238.

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10.5

Hydrolysis of Biomass by Sulphur Dioxide

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ABSTRACT

Results of hydrolysis of several types of cellulosic biomass, namely pine sawmill residues, aspen poplar chips, sugarcane bagasse, wheat straw and corn cobs, are presented in which sulphur dioxide was the hydrolysis catalyst. Hemicellulose was first hydrolysed at 150°C, and following removal of hemicellulose sugars by water washing, residual cellulose was hydrolysed at 190°C, using SO_2 in both stages. Hemicellulose hydrolysis with SO_2 catalyst (prehydrolysis) was compared with steam hydrolysis in the absence of added catalyst (autohydrolysis) and poplar. In SO_2 hydrolysis of these five biomass species, recovery of hemicellulose sugars was essentially quantitative, while cellulose hydrolysis varied from 54·5% for wheat straw to 84·3% for pine. Some economic consequences of this process are discussed. Yields of ethanol, butanol and furfural from these raw materials were estimated.

Key words: acid hydrolysis, cellulosics, wood, bagasse, straw, corn cobs, autohydrolysis, ethanol, butanol, furfural.

INTRODUCTION

Presently operating fermentation fuel alcohol plants in Brazil and in the USA are based on sugar cane byproducts or on corn, which are both human food or animal feed, and there is widespread concern about driving automobiles on food- or feed-based fuel. This conflict is heightened by the need for relatively good land for these crops, resulting in competition for land. Inevitably, it is feared, such competition will

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raise food costs, with harmful social consequences. As a result, much attention has been devoted to the much more difficult problem of converting cellulosics – wood, agricultural crop residues – to power alcohols.[1]

In Canada now there is enough waste wood and waste crop residual surplus to the needs of soils and animals, and in relatively concentrated locations to make about 6 billion litres/year of fermentation fuel ethanol, which is about 20% of Canadian gasoline consumption.[2] In addition, there is available abandoned farmland and other poor land on which to plant fast-growing hybrid trees and other energy crops sufficient to supply all of the rest of the motor fuel Canada requires. It would need only 5 million ha of relatively poor land to provide the cellulosic raw material for the full amount of motor fuel.

All presently operating commercial plants for producing fermentable sugars from wood have been based on hydrolysis by dilute sulphuric acid in percolation towers. Such processes have poor yields and many operating problems;[3] they give rise to humic substances which inhibit fermentation[4] and severe corrosion. Furthermore, the economics of such plants are questionable.[5]

A hopeful improvement appeared with the introduction of autohydrolysis as a pretreatment for cellulosics.[6] Autohydrolysis, that is steam cracking followed by rapid quenching, is highly effective for hydrolysis of hemicelluloses of deciduous woods, such as poplar, and of agricultural crop residues. In addition, much of the lignin becomes soluble in dilute alkali, or in organic solvents, and extraction of the lignin leaves a relatively pure cellulose, for hydrolysis or for other uses. The autohydrolysis-extraction-hydrolysis process[7] results in good yields of fermentable sugars and ethanol.[8] However, until now this process has not proved useful for coniferous woods such as pine, spruce or hemlock, which are the main residues of the forest industries. To improve the process by extending its raw material base to include the conifers, and to find a satisfactory alternative to sulphuric acid as hydrolysis catalyst, we have compared several acids as hydrolysis catalysts for production of fermentable sugars from cellulosics.[9] Of these, sulphur dioxide proved to be the most effective. Sulphur dioxide has a number of specific advantages as hydrolysis catalyst over H_2SO_4. Since SO_2 is a gas, it can be added either ahead of or with the steam; it is better distributed through the biomass than H_2SO_4, resulting in a more uniform reaction. Less SO_2 is used than H_2SO_4, and it costs less.

SO_2 has been made and used for many years in the pulp and paper industry in pressure vessels, and stainless steels are available which are satisfactorily resistant to attack by aqueous SO_2, while metallurgy for H_2SO_4 at these temperatures remains a serious problem. Lignin remains very light in colour in SO_2 hydrolysis, especially if sulphate or oxygen scavengers are present, whereas it turns black during sulphuric acid processing. Lignosulphonates made by the reaction of SO_2 during sulphite pulping of wood are the basis of a substantial chemical industry, including production of vanillin. Preliminary work here suggests that these lignins will prove to be quite suitable for chemical conversion.

BIOMASS ANALYSES

The biomass species examined were: pine, mainly red pine with some white pine, as sawmill residues; aspen poplar chips about 3 X 12 X 20 mm; bagasse from a cane sugar mill in Louisiana; corn cobs from a large corn starch and alcohol plant; and wheat straw from a nearby farm. The summative analyses are given in Table 1.

TABLE 1
Summative Analysis of Cellulosics (% of dryweight)

	Red pine sawmill residue	*Aspen chips*	*Bagasse Louisiana*	*Corn cobs*	*Wheat straw*
Polysaccharides	68·0	67·7	59·9	76·3	73·0
Klason lignin	25·8	21·5	25·0	9·7	14·1
Acid-soluble lignin	1·2	5·4	1·6	3·2	2·0
Extractives	5·0	5·4	4·0	3·2	4·5
Ash	–	–	2·7	0·7	6·4
Other	–	–	6·8	6·9	–
	100·0	100·0	100·0	100·0	100·0
Polysaccharides					
Glucan	50·7	42·2	34·9	32·0	37·5
Non-glucan	17·3	25·5	25·0	44·3	35·5

COMPARISON OF AUTOHYDROLYSIS AND PREHYDROLYSIS WITH SO_2

Samples of the pine and the aspen woods were heated in small autoclaves in the presence of a small amount of water for autohydrolysis, or with very dilute SO_2 solution for prehydrolysis. The time and temperature of autohydrolysis were 15 min at 190°C and of prehydrolysis were 20 min at 150°C. The results are shown for pine in Table 2 and for aspen in Table 3. They confirm[5] that autohydrolysis is much more effective in hydrolysing aspen hemicellulose than it is pine hemicellulose. In the case of aspen, 88·4% of the theoretical yield of non-glucose sugars was obtained on autohydrolysis, whereas for pine only 59·2% of the hemicellulose non-glucose sugars was hydrolysed to soluble carbohydrates. A large proportion of these sugars occurred as oligomers rather than monomers, about 37% of the pine sugars and about 42% of the aspen sugars.

TABLE 2
Autohydrolysis and Prehydrolysis of Pine

	Glucose (%)	*TRS (%)*	*Fibre residue*	*Alkali extract*
Autohydrolysis (steam), 190°C, 15 min				
Monomer	1·52	11·78	74·33	9·22
Oligomer	2·32	3·32		
Total	3·84	15·10		
% of theory				
Glucose	6·9			
Non-glucose	59·2			
Prehydrolysis, 2% SO_2: wood, 150°C, 20 min				
Monomer	5·29	20·55	69·06	4·91
Oligomer	–	4·32		
Total	5·29	24·87		
% of theory				
Glucose	9·5			
Non-glucose	102·9			

TABLE 3
Autohydrolysis and Prehydrolysis of Aspen

	Glucose (%)	*TRS (%)*	*Fibre residue*	*Alkali extract*
Autohydrolysis (steam), 190°C, 15 min				
Monomer	1·41	15·36	69·1	14·40
Oligomer	1·47	11·00		
Total	2·88	26·36		
% of theory				
Glucose	6·2			
Non-glucose	88·4			
Prehydrolysis, 3% SO_2: wood, 150°C, 20 min				
Monomer	2·56	29·07	67·1	7·86
Oligomer	0·81	1·62		
Total	3·37	30·69		
% of theory				
Glucose	7·8			
Non-glucose	102·0			

In the presence of a small amount of SO_2 catalyst, 2% on pine wood (dry basis, 0·5% SO_2 solution) and 3% on aspen wood (dry basis, 0·75% SO_2 solution), hydrolysis of hemicellulose took place at much lower temperature (150°C instead of 190°C) and was complete. The sugars occurred mainly as monomers, only 5·3% of the aspen hemicellulose sugars being oligomers and 17% of the pine hemicellulose sugars.

It was noted that the solubility of the lignin in alkali was reduced on SO_2 prehydrolysis being about half that in autohydrolysis in both cases, presumably due to acid-catalysed polymerization.

TWO STAGE SO_2 HYDROLYSIS

The procedure adopted for hydrolysis of these cellulosics was SO_2 prehydrolysis for 20 min at 150°C, the amount of SO_2 being 2–3% on fibre, in 0·5 or 0·75% SO_2 solution; the hemicellulose sugars were then

washed from the fibrous residue with water and the alkali-soluble lignin with 2% NaOH; finally the fibrous residue was hydrolysed with 1% SO_2 on fibre (0·25% solution) at 190°C. The hydrolysis procedure was adapted to the particular small reactors used, such that temperature was raised during 16 min to 190°C and held there for 4 min, whereupon the reactor was cooled rapidly, and the glucose washed from the fibrous residue. This heating, cooling and washing was repeated a total of four times. This rather awkward procedure was adopted in order to minimize sugar destruction by the acid at the high temperature.

The results are shown in Table 4. As expected, hemicellulose hydrolysis was essentially complete in all cases. However, the amount of sugar recovered from the residual cellulose varied widely among these five species of biomass. The procedure was very good for pine wood, 84·3% of the theoretical yield of glucose being obtained. All other glucose yields were less than 70%, varying from 68·7% for aspen to only 54·5%

TABLE 4
Sugar Yields on Two-Stage SO_2 Hydrolysis of Cellulosics (% of dry raw material weight)

	Red Pine sawmill residue	*Aspen chips*	*Bagasse Louisiana*	*Corn cobs*	*Wheat straw*
Prehydrolysis					
Glucose	6·9	3·3	3·0	3·0	2·5
Non-glucose sugars	21·1	23·3	24·8	42·9	43·8
Hydrolysis					
Glucose	40·1	28·9	20·4	20·5	20·2
Non-glucose sugars	1·0	3·3	4·0	7·1	2·0
Total					
Glucose	47·0	32·2	23·4	23·5	22·7
Non-glucose sugars	20·1	26·6	28·8	50·0	45·8
% of theoretical yield					
Glucose	84·3	68·7	60·4	66·2	54·5
Non-glucose sugars	103·7	91·5	101·0	99·0	113·2

for wheat straw. Since in all cases the amount of fibrous residue at the end of the process was about what would be expected from the lignin content, the difference is due not to differences in rate of hydrolysis, but rather to differences in the rates of destruction of the glucose as formed; or perhaps a better formulation might be in our failure to preserve the glucose as formed. This procedure obviously requires improvement. However, it can be pointed out that the yield reported here for glucose from pine may be the highest yet obtained.

ALCOHOLS AND FURFURAL FROM CELLULOSICS

Based on the yields of hexose and pentose sugars given in Table 4, yields of ethanol, butanol–acetone–ethanol and furfural have been estimated and are listed in Table 5. Ethanol yields are best with pine by some significant margin, based on fermentation of hexose sugars only (glucose and mannose) by ordinary yeast (*Saccharomyces cerevisiae*). No allowance has been made for possible fermentation of pentoses by *Paelysolen tannophilus*, or for conversion of pentose sugars to the pentulose form by isomerase and subsequent fermentation, two processes under investigation for fermentation of pentose sugars to ethanol.

Fermentation by *Clostridium acetobutylicum* to butanol–acetone–ethanol solvents would make use of both hexose and pentose sugars. Pine hydrolysates would give the highest yields of these solvents (fuels);

TABLE 5

Chemical Yields (litres Mg^{-1} dry raw material) on Two-Stage SO_2 Hydrolysis of Cellulosics (estimated from sugar yields)

	Red pine sawmill residue	*Aspen chips*	*Bagasse Louisiana*	*Corn cobs*	*Wheat straw*
Ethanol	475 ~~324~~	470 ~~185~~	415 ~~135~~	530 ~~125~~	505 ~~130~~
Butanol-acetone-ethanol	252	220	196	276	257
Furfural	28	73	80	138	126

however, yields from hydrolysates of the other species of biomass are estimated to be quite high, with corn cobs and wheat straw having an advantage compared to bagasse or aspen. Corn cobs and wheat straw are also the best sources of furfural, according to these estimates. The bagasse came out relatively poorly as a furfural source, which is surprising since there is a commercial operation producing furfural from sugarcane bagasse in southern Florida.

ECONOMICS

Based on earlier economic studies,[5] it is possible to estimate capital and operating costs of plants to make ethanol based on these results with the pine sawmill residues. Considering a plant to make 240 000 litres day^{-1} of anhydrous ethanol (84 million litres $year^{-1}$), investment is estimated at Cdn $31·2 million, or 37·1 ¢ $litre^{-1}$, before return on investment. Return on investment of 20% would amount to a further 7·4 ¢ $litre^{-1}$. This figure can be considered the approximate cost of gasoline at the refinery gate, so the two are competitive. Any reduction in wood costs would improve its competitive position, or perhaps permit a more attractive return on investment.

The above calculation includes no subsidy or tax concession, both of which are common in the fuel industry. Also, no allowance has been made in this calculation for the sale of lignin or other byproducts. Any substantial sale of lignin above its fuel value would materially improve the economics of processing pine wood to ethanol.

To comment briefly on the other biomass species considered here, none of them is as attractive as pine for ethanol production. This is primarily due to an unfavourable hexose/pentose balance. The finding of a successful process for fermentation of pentoses to ethanol would improve the economics materially for these other species. This is shown clearly in the estimates of butanol solvent production, where the advantage of pine over the other species is not so marked. Interest in butanol has increased quite markedly recently because of its use as a co-solvent in methanol–gasoline blends. In these fuels, the methanol is derived from natural gas, and up to now the butanol has been derived from petrochemical processes. However, newer processes[10] suggests that fermentation butanol may be competitive with petrochemical butanol.

REFERENCES

1. *Proceedings, international symposium on ethanol from biomess*, Royal Society of Canada, Winnipeg (1982).
2. Hayes, R. D. (1982). Ref. 1, pp. 118–41; Clarke, M. R. & Sastry, C. B. R. (1982). Ref. 1, pp. 142–60.
3. Burton, R. J. (1982). Ref. 1, pp. 247–70.
4. Mackie, K., Deverell, K & Callander, I. (1982). Ref. 1, pp. 271–311.
5. Wayman, M. (1983). *Assessment of the New Zealand Forest Research Institute process for pine wood hydrolysis and fermentation to ethanol*, Report to Energy, Mines and Resources Canada.
6. Lora, J. H. & Wayman, M. (1978). *Tappi*, 61(6), 46–50; Wayman, M. & Lora, J. H. (1983). Canadian Patent 1,147,105; Okamura, K. & Morikawa, H. (1983). In: *Recent technologies and prospects of wood chemicals*, ed. J. Nakano and T. Higuchi, CMC Report No. 40, Tokyo, CMC Publishers.
7. Wayman, M. (1980). *Proceedings IV intern. alcohol fuels techn. symp.*, Vol. I. pp. 79–84.
8. Wayman, M., Lora, J. H. & Gulbinas, E. (1978). In: *Chemistry for energy*, ACS Symposium Series Monograph No. 90, pp. 183–201.
9. Wayman, M. & Tallevi, A. (1983). ACS Annual Meeting, Washington, DC.
10. Wayman, M., Husted, G., Santangelo, J. & Levy, S. (1984). Paper to be presented at VI Intern. Alcohol Fuels Techn. Symp., Ottawa.

Hydrolysis of Biomass by Sulphur Dioxide
2. Recalculation of Ethanol Yields Including Pentose Fermentation

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ABSTRACT

Yields of ethanol calculated from the sugars obtained by two-stage SO_2-catalysed hydrolysis of biomass are now much higher than reported earlier (1) as a result of successful rapid and efficient fermentation of xylose to ethanol by R strains of Candida shehatae and Pichia stipitis (2). Increases in ethanol yields due to xylose fermentation were calculated, based upon the quantities of hexose and pentose sugars obtained upon SO_2 hydrolysis, to be: for pine wood 27.8%, aspen 42.7%, bagasse 52.6%, corn cobs 65.8% and wheat straw 64.6%. Ethanol yields calculated from sugar yields were 405 to 433 LMg-1 from wheat straw, pine and corn cobs, 356 from aspen and 312 from bagasse.

Key words: acid hydrolysis, cellulosics, wood, bagasse, straw, corn cobs, ethanol, xylose, fermentation.

In an earlier paper[1] the author and colleagues described the SO_2-catalysed hydrolysis of 5 different kinds of biomass, pine and aspen woods, sugar cane bagasse, corn cobs and wheat straw. From the sugar yields obtained on two-stage SO_2 hydrolysis of these cellulosics, yields of ethanol were calculated as LMg-1 (Reference 1, Table 5). These yields were based on fermentation of hexose sugars only by ordinary yeast Saccharomyces cerevisiae, and no allowance was made for possible fermentation of pentoses by Pachysolen tannophilus, or for conversion of pentose sugars to the pentulose form by isomerase and subsequent fermentation. Recently, the author and colleagues have had remarkable success in fermenting xylose rapidly and efficiently by R strains of Candida shehatae and Pichia stipitis[2]. These strains, generated by repeated recycling under conditions of some stress, also have high sugar and alcohol tolerance[3]. This finding requires the recalculation of the yields of ethanol to be expected from fermentation of the sugars obtained by two-stage SO_2 hydrolysis of these cellulosics.

Ethanol yields are calculated by multiplying the glucose yields reported in Table 4 of Reference 1 by 0.50 and the yields of non-glucose sugars from the same table by 0.45, and then dividing the products by 0.79 to convert weight percentages to volume percentages. The hexose ethanol yield, which in theory should be 0.51,

has been lowered to allow for the presence of galactose which, in our experience in fermentation of wood hydrolysates, ferments slowly. The non-glucose ethanol yield has been lowered from the theoretical 0.465 obtainable from pentoses to allow for the presence of arabinose. Arabinose, we have found, is not fermented to ethanol by the R yeasts, but serves as maintenance nutrient, about half the arabinose present being assimilated for that purpose.

The newly calculated ethanol yields are shown in Table 1.

TABLE 1

Ethanol Yields (LMg-1 dry biomass) on Two-Stage SO_2 Hydrolysis of Cellulosics, Calculated from Sugar Yields Reported in Reference 1

Sugar Source	Red Pine Sawmill Residue	Aspen Chips	Bagasse Louisiana	Corn Cobs	Wheat Straw
Glucose	298	204	148	149	144
Non-glucose Sugars	115	152	164	285	261
Total	413	356	312	434	405
Xylose Contribution as Percent of Total	27.8	42.7	52.6	65.8	64.6

In the earlier paper[1], pine wood was reported as the best source of ethanol from this group of biomass raw materials, while the others gave lower yields by a considerable margin. The ability to ferment xylose, which is an important constituent of decicuous woods

such as aspen and of agricultural residues, has raised the yields of ethanol to be expected from corn cobs and from wheat straw to about the same level as that from pine, and while the yields to be expected from aspen and from bagasse have been raised considerably, they are still below that from pine. In all cases, the contribution from xylose fermentation to ethanol production is highly important, ranging from 27.8% from pine to 65.8% from corn cobs.

REFERENCES

1. Wayman, M., Tallevi, A., and Winsborrow, B. (1984). Biomass 6, 183-191.

2. Parekh, S., Yu, S., and Wayman, M. (1986). J. Appl. Microbiol. Biotechn., in press.

3. Wayman, M. and Parekh, S. (1985). Biotechn. Letts. 7, 909-912.

Acid Hydrolysis of Lignocellulosics for Alcohol Production

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Abstract

Slurries of agricultural wastes have been successfully prehydrolyzed and delignified using the St. Lawrence pilot plant continuous plug flow reactor. Conditions established for essentially complete recovery of the hemicellulose component of corn cobs as the monosaccharides xylose and arabinose are approximately 7 minutes at 150°C with 0.5% HCl. Delignification of prehydrolysed corn cobs with 4% alkali at 167°C for 8 minutes resulted in more than 99% of the lignin being removed. Of the lignin removed, about 80% was recoverable. The remainder maintained its solubility even after acidification. The yeast Candida shehatae successfully fermented pure xylose and glucose but was not as effective at fermenting corn cob prehydrolyzates. Single pass hydrolysis of Alpha Floc has thus far resulted in a maximum glucose yield of 45%. However, initial hydrolysis of prehydrolysed and delignified corn cobs has resulted in a 60% glucose yield. Preliminary cost estimates indicate that under certain assumptions a 40 million L/yr plant can produce ethanol at a selling price of 45¢/L.

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1. Introduction

In the recent past the economy of the world has been undergoing a structural shift from overwhelming dependence on nonrenewable resources to one in which renewable resources are playing an ever increasing role. As such, more and more research is being devoted to developing technologies that will produce biomass derived fuels and chemicals cost-effectively with those from oil. The plunge in world oil prices that has recently rocked the world should not deter from this effort.

One particular chemical that has captured the attention of both past and present researchers is ethanol. Although utilization of starch for conversion to ethanol has been firmly established and has found its own particular niche in today's world economy, the same cannot be said of cellulose. Cellulose is the world's most abundant renewable resource and is the major component of most biomass. Economic viability of any future commercial process lies not in focusing solely on cellulose, but rather, in utilizing the complete biomass feedstock. This includes generating revenue from both the hemicellulose and lignin streams. In the envisaged St. Lawrence process, the hemicellulose is hydrolyzed for subsequent fermentation to ethanol while the lignin is isolated and recovered as a value-added co-product. A process flowsheet is shown in Figure 1.

St. Lawrence Reactors Limited is a technical development company which markets, develops and conducts research on all conversion of biomass. It has commercialized starch conversion technology and has designed, built, installed and started up large scale plant equipment for corn syrup and fermentation substrate production. This paper will describe the ongoing research efforts of St. Lawrence Reactors in the area of acid hydrolysis of lignocellulosics.

2. Materials and Methods

St. Lawrence Reactors operates a continuous 15 L/min (four GPM) plug flow acid hydrolysis reactor. The reactor consists of 1" tubing and is heated indirectly with steam. Variable residence times can be attained by rearranging the tubing configuration (adding or subtracting tubing) or by changing the speed of the pump. A Robbins and Myers 9P4 progressing cavity Moyno feeding pump is used. An interchangeable orifice maintains back pressure within the reactor. For cellulose hydrolysis the accompanying boiler can generate 600 psig (254°C) steam.

Sugar concentrations were determined by HPLC using a differential refractometer detector, a Waters M-45 solvent delivery system, a Waters U6K universal injector and a Bio-Rad HPX-87-H organic acids column. Column temperature was 30°C using a mobile phase of 0.01N H_2SO_4 and a flowrate of 0.6 mL/min. Quantitation was performed using the external standard method and multi-level calibration.

3. Results

3.1 Feedstock Composition

The results of the analysis of four feedstocks are summarized in Table 1.

3.2 Prehydrolysis

Corn cobs were chosen as the feedstock to be most extensively examined for determination of conditions necessary for maximum recoveries of hemicellulose. Numerous first stage pilot plant hemicellulose hydrolysis runs were performed on the corn cobs at various temperatures and acid loadings and at more or less constant residence times. Several of these results are summarized in Table 2 and plotted in Figures 2 and 3.

Figure 2 is a plot of the combined xylose and arabinose yield as a function of acid loading. The ordinate scale is simply the combined mass of xylose and arabinose divided by the total mass of dry corn cob material fed into the reactor.

Figure 3 shows glucose yield as a function of acid loading. The ordinate scale is, as before, simply the mass of glucose divided by the mass of original dry corn cob material fed to the reactor.

A compositional analysis of the corn cobs revealed a hemicellulose content of 24% and a starch content of 22%. If all of this hemicellulose is converted to arabinose and xylose, a total five carbon sugar yield of about 27% would be expected; similarly if all of the starch is converted to glucose, a glucose yield of about 24% should be obtained. Together the first stage maximum total sugar yield should be in the order of 51%. Table 2 shows that at all of the three temperatures examined (with residence times ranging from 6 to 8 minutes), the maximum total sugar yield always exceeds 51%. The maximum at 140°C is lower than at both 150°C and 167°C; the maximum at 150°C is in turn lower than at 167°C. The differences in Table 2 between the total sugar yields and the sum of the glucose, xylose and arabinose sugar yields represents sugars that are dimers, trimers and higher degrees of polymerization (DP).

Although maximum total sugar yields occur at 167°C, maximum yields of hemicellulose sugars are obtained at 150°C. The maximum yield is about 32% which is higher than the theoretical amount of 27% based on the compositional analysis of the corn cobs. This clearly shows that the analysis employed does not account for all of the hemicellulose or alternatively, that the prehydrolysis results in a

substance eluting on the HPLC (High Performance Liquid Chromotograph) at the same retention time as xylose or arabinose. At 167°C the C_5 sugar yield is about 30.5% and at 140°C is about 29.5%. The maximums all occur at acid strengths of about 0.51% (slightly higher for the 140°C maximum). At a feed solids level of 15% (all of the runs were done using a feed solids loading of around 15%) this represents about 2.9% acid on fibre. A calibration curve relating acid strength to pH for corn cobs at 18% dry solids reveals that 0.51% acid corresponds to a pH of about 1.3.

From Figure 3 the maximum glucose yield occurs at 167°C. The maximum glucose yield at 140°C is lower than at 150°C, which in turn is lower than at 167°C. The maximum glucose yield of 20% at 167°C is less than the theoretical amount of 24% based on the compositional analysis and is due to the fact that some of the starch remains as dimers, trimers and higher oligomers. The maximum glucose yield at 150°C is about 18% and at 140°C is about 15%. The acid loading necessary to achieve the maximum glucose yield at any given temperature increases with decreasing temperature. At 150°C the acid strength at the maximum is around 0.54%. Since the optimal temperature for obtaining xylose and arabinose is 150°C at an acid strength of 0.51%, this will result in slightly less than the optimum yield of glucose. However, Figure 3 shows that the maximum yield is not highly sensitive to acid loading and decreasing from 0.54% acid to 0.51% acid results in a drop in glucose yield of only about 0.5%.

Although the yield of xylose and arabinose on the original hemicellulose present in the corn cobs, as determined by compositional analysis, is greater than 100% (as high as 118%), if a literature value of 29% hemicellulose in corn cobs is used, (from Physical Properties, Chemical Properties and Uses of the Andersons' Corncob Products by Dr. Kevin M. Foley) the yields become much more reasonable and decrease below 100%. The maximum yield of xylose and arabinose based on available hemicellulose now becomes 98%.

Analysis of the prehydrolyzed pulp reveals that it contains significant amounts of polymeric hemicellulose. In one particular prehydrolysis, calculations based on a residual pulp analysis revealed that 86% of the original hemicellulose had been converted. This contrasted with a figure of about 119% based on HPLC analysis of the aqueous solution of solubilized sugars. If the 9.7% of missing material obtained from the compositional analysis of the original corn cobs (see Table 1; Batch #3) is assumed to be all hemicellulose, the recovery based on HPLC analysis for solubilized sugars drops to 88%. The remaining polymeric hemicellulose would now account for about 11% of the original hemicellulose. This closed mass balance around the hemicellulose may indicate that, for some reason, the methods employed in the compositional analysis do not effectively convert all of the hemicellulose to constituent monosaccharides. Since the method used quantitates these monosaccharides to back calculate the original amount of hemicellulose in the corn cobs, the resulting hemicellulose contents will be low. If this postulate is true, the same problem appears not to manifest itself when analyzing the prehydrolysed product pulps.

In conclusion it can be stated that essentially all of the hemicellulose in corn cobs can be recovered as the monomers xylose and arabinose when the following optimal conditions are used:

Temperature	$\sim$ 150°C
Acid Strength	$\sim$ 0.51%
Time	$\sim$ 7 minutes

At the above conditions approximately 70% of the starch will also be recovered as glucose.

Other feedstocks such as wheat straw, Jerusalem Artichoke tops and wood have since been prehydrolyzed with yields ranging from only 68% with aspen wood to essentially 100% with both wheat straw and Jerusalem Artichoke tops. Work is continuing in this area to determine if the conditions used for the corn cobs will result in the same high yields of five carbon sugars from these other feedstocks.

3.3 Delignification

An alkali extraction method is currently being employed for removal of lignin. The pilot plant reactor is used for this purpose at the following operating conditions:

Temperature:	~	167°C
Caustic Strength:	~	4%
Time:	~	8 minutes

At these conditions approximately 78% of the original lignin in corn cobs can be recovered. Recovered lignin is lignin that once solubilized in caustic solution can be precipitated upon acidification. Less than 1% of the original lignin remains behind in the fiber. Presumably the remaining 20% or so of the lignin remains solubilized. Soluble lignin determinations by UV methods verified the presence of lignin in the acidified solution.

Samples of the isolated corn cob lignin have been submitted for manufacture of phenol-formaldehyde resins, for vanillin production and for conversion to diesel fuel. Preliminary results have shown that the lignin has performed very well in an oxidative process yielding diesel-like fuel.

Experience acquired to date on delignification reveals that agricultural materials such as corn cobs and wheat straw are far easier to delignify than wood.

A mass balance flowsheet for processing corn cobs through the first two stages (prehydrolysis and delignification) is shown in Figure 4.

3.4 Fermentation

The ability of the yeast strain Candida shehatae to ferment xylose is shown in Figures 5A and 5B. At concentrations up to 90 g/L of xylose (at dry solids levels of 20% and a feedstock composition of 25% hemicellulose, the maximum xylose concentration would be about 60 g/L), the yeast is able to completely ferment the sugar in 70 hours or less. The corresponding yield of ethanol is about 0.37 g EtOH/g sugar. The theoretical yield is 0.47g EtOH/g sugar consumed.

Figures 6A and 6B show the ability of Candida shehatae to simultaneously ferment both glucose and xylose. With 70:30 xylose/glucose mixtures and total sugar concentrations up to 150 g/L (105 g/L xylose), the yeast is able to completely ferment the sugars in less than 60 hours. Assuming a 100% efficient fermentation of glucose, the fermentation yield from xylose is about 0.39 g EtOH/g xylose. This value is close to the yield from Figures 5A and 5B.

To date, the greatest number of fermentations have been performed on corn cob prehydrolyzates. Some of these are summarized below in Table 3. From this data it becomes evident that the yeast is not as effective at fermenting these prehydrolyzates as pure sugar solutions. This may indicate that the yeast cannot perform in the presence of impurities and/or salts.

Another possibility concerns the presence of oxygen. Although xylose is being consumed, it is not being converted to ethanol. These fermentations were done under semi-aerobic conditions and although Candida shehatae follows an oxidative pathway to ethanol, excessive oxygen will result in yeast growth and glycerol production. Glycerol was detected in the final fermentation broths at concentrations up to about 2.9 g/L. It is the high osmotic pressure due to salts that initiates glycerol production by the yeast as a defensive mechanism. This is another reason that some oxygen is desirable. However, the fermentations of pure xylose and glucose shown in Figures 5A, 5B, 6A and 6B were all performed under the same semi-aerobic conditions as the corn cob prehydrolyzates. This indicates that the salt present in the latter but not the former may be the reason for the inefficient fermentations.

It should also be appreciated that the fermentation efficiency of xylose would increase if the fermentation of glucose was not 100% efficient. Since it appears as if some xylose is being utilized for yeast growth, it is not unreasonable to expect that some glucose was also consumed for this purpose and not converted to ethanol.

3.5 Hydrolysis

The results of single pass hydrolysis of Alpha Floc in the St. Lawrence pilot plant reactor, are shown in Table 4. From the data, the highest glucose yield of 45% is obtained at 238°C with an acid strength of 0.15% and a residence time of 1.6 minutes (AF 2-2b). Decreasing the time to only 0.8 minutes at the same acid loading results in the glucose yield dropping to 42% (AF 2-2a). The yield of non-volatiles, which can be assumed to include some unreacted cellulose, correspondingly increases from 26% to 40%. The same conditions at 218°C results in a 27% glucose yield at a 1.6 minute residence time (AF 2-1b) and 24% at 0.8 minutes (AF 2-1a). Increasing the temperature to

252°C with the same acid strength of 0.15% and residence times of 1.6 minutes and less than 1.6 minutes decreases glucose yields to only 3.9% and 5.3% respectively. At temperatures as high as 254°C, residence times obviously must be less than 1.6 minutes in order to obtain reasonable yields of glucose. However, both heat transfer and mechanical considerations preclude obtaining shorter residence times at these temperatures (AF 2-6a, AF2-6b).

With 80' of coil and 0.15% acid, the optimum boiler temperature setting lies between 220°C and 254°C. At a boiler temperature of 240°C, increasing the coil length to 120' and correspondingly increasing the velocity to maintain the same residence time, results in a drop in the glucose yield from around 45% (AF 2-2b) to about 25% (AF 2-5). Compared to 233°C with AF 2-2b, the temperature after 40' in the AF 2-5 run is only 173°C. At 80' the temperatures are 238°C for AF 2-2b and 230°C for AF 2-5. Over the last 40' the temperature with AF 2-5 increases to 238°C. The net effect of the slower heat up time in AF 2-5 (as a result of a higher velocity) is that the total thermal input is greater in the AF 2-2b run than in the AF 2-5 run. The cellulose spends more time at higher temperatures in the AF 2-2b run; this despite both runs having the same residence time, temperature and acid strength. This is the reason the conversion to glucose is higher in AF 2-2b than in AF 2-5.

There is, however, somewhat of a discrepancy with the yield from AF 2-5 being as low as 24.8%. The decrease in glucose yield between AF 2-1b (27%) and AF 2-4 (25%) is not as great as the difference between AF 2-2b (42%) and AF 2-5 (25%). This can be explained by the fact that the reduction in thermal inputs (due to the increased velocities) for the AF 2-4 and AF 2-5 runs will be much more significant at the higher boiler temperature of 240°C used for AF 2-5 (see temperature profiles; AF 2-1b vs AF 2-4 and AF 2-2b vs AF 2-5). Nevertheless, the yields for AF 2-4 and AF 2-5 should not be similar. The AF 2-5 yield would be expected to be lower than that from AF 2-2b, but at the same time higher than AF 2-4. A yield of around 30% would be more reasonable. Preliminary fermentation data

also indicate that the extent of reaction was greater in AF 2-5 than in AF 2-4 since the former did not ferment at all whereas the latter did, albeit slowly. This would be consistent with more yeast inhibitors being present with the harsher reaction conditions of AF 2-5. It may be that the 23% yield figure is a bad data point.

The conditions used in AF 2-2b have thus far given the highest yields of glucose. However, this is not to imply that they are optimal. Further work can be done to see if improvements on the 45% yield can be obtained. The residence time can be increased or decreased to see if a maximum occurs somewhere between 0.8 minutes and 1.6 minutes or if it occurs beyond 1.6 minutes. However, as mentioned earlier, adjusting the residence time will also affect the temperature profile and this effect must be considered. It is these nonisothermal conditions that render interpretation of results more difficult and the implementation of reaction kinetics more complicated than if classical isothermal reaction conditions had prevailed.

Preliminary hydrolyses with isolated corn cob cellulose have resulted in glucose yields as high as 60%.

4. Economics

The economics of producing ethanol from waste wood have been considered. Two plant capacities have been selected for performing these preliminary economic calculations. The first is 20 million L EtOH/yr (Case A) and the second, 40 million L EtOH/yr (Case B). Two scenarios have also been included. The first assumes no recovery of the delignification chemicals and the second assumes a 90% recovery.

The following assumptions have been made:

1) Wood cost: i) Hardwood delivered chips: $49.60/oven dry tonne
ii) Softwood delivered sawdust: $14.30/oven dry tonne

2) Wood Composition:

i) Hardwood:	Cellulose	50%
	Hemicellulose	24%
	Lignin	22%
ii) Softwood:	Cellulose	45%
	Hemicellulose	20%
	Lignin	30%

3) Plant processes a 50:50 mix of hardwood and softwood.

4) Softwood hemicellulose is 35% C5's and 65% C6's.

5) Hardwood hemicellulose is 90% C5's and 10% C6's.

6) Recovery of hemicellulose as fermentables of 85% and a fermentation efficiency of 85%.

7) Recovery of cellulose as fermentables of 65% and a fermentation efficiency of 90%.

8) Recovery of 60% of the lignin.

9) Value assumed for recovered technical grade lignin: i) Low:14¢/lb
ii) High:20¢/lb

10) Yeast propagation will be incorporated into the plant.

11) All fiber streams are processed at 10% dry solids.

SUMMARY OF ANNUAL PRODUCTION COSTS ($000)

	Case A	Case B
Wood	2,200	4,300
Chemicals: HCl Prehydrolysis	1,300	2,600
Hydrolysis	100	200
Delignification:		
No Recovery: NaOH	7,700	15,400
: H_2SO_4	4,300	8,600
90% Recovery: NaOH	800	1,500
: H_2SO_4	400	900
Yeast (with propagation system)	200	400
Energy (with heat recovery)	3,000	5,900
Labour and Management	1,000	1,500
Repair and Maintenance (4% of capital cost)		
No Recovery	500	800
90% Recovery	600	1,000
Taxes and Insurance (2% of capital cost)		
No Recovery	300	400
90% Recovery	300	500
Return on Investment (35% of capital cost)		
No Recovery	4,600	7,300
90% Recovery	5,300	8,500
TOTAL ANNUAL COST:		
No Recovery:	**25,200**	**47,400**
90% Recovery:	**15,200**	**27,300**

BY-PRODUCT CREDITS ($000)

Lignin

	Low Lignin Price	High Lignin Price
Case A	3,300	4,700
Case B	6,600	9,400

NET ANNUAL COST BEFORE REVENUE FROM ETOH ($000)

	CASE A		CASE B	
	Low Lignin Price	High Lignin Price	Low Lignin Price	High Lignin Price
NO RECOVERY	25,200	25,200	47,400	47,400
	-3,300	-4,700	-6,600	-9,400
	21,900	20,500	40,800	38,000
90% RECOVERY	15,200	15,200	27,300	27,300
	-3,300	-4,700	-6,600	-9,400
	11,900	10,500	20,700	17,900

ETOH SELLING PRICE ($/L)

	CASE A		CASE B	
	Low Lignin Price	High Lignin Price	Low Lignin Price	High Lignin Price
NO RECOVERY	1.10	1.03	1.02	0.95
90% RECOVERY	0.60	0.53	0.52	0.45

5. Conclusions

Conditions for efficient prehydrolysis and recovery of the hemicellulose component from agricultural materials have been firmly established. Lignin has also been successfully isolated in good yields using an alkali treatment.

Further work is planned in improving the fermentation of the prehydrolyzates, obtaining conditions for maximum glucose yields in the hydrolysis step and extending all of the aforementioned work to wood.

Efforts are also being directed at altering the feeding mechanism to the reactor so that feed slurries with higher dry solids levels can be processed.

Funding for this work from Agriculture Canada under the ERDAF program as well as from the Ontario Ministry of Energy is gratefully acknowledged.

TABLE 1

Feedstock Compositions

	Corn Cobs		Wheat Straw	Jerusalem Artichoke Tops	Pine Wood	Aspen Wood
	Batch#2	Batch#3				
Cellulose	24.1	33.2	21.8	18.3	48.6	52.1
Hemicellulose	24.0	27.8	20.9	12.0	21.8	21.1
Lignin	13.5	12.5	21.9	27.7	29.6	22.2
Starch	22.1	10.3	10.5	12.0	--	--
Protein	5.6	3.9	6.5	6.0	--	--
Extractives	1.3	0.6	1.5	0.9	5.2	5.5
Ash	3.4	2.0	6.6	2.4	0.5	0.3

TABLE 2: Corn Cob Prehydrolysis Optimization Runs

Run Number	Temp (C)	Time (min)	Feed D.S. (%)	Acid Strength (%)	Sugar Yields (% on Feed D.S.) Total Sugar	Glucose	Arabinose & Xylose
CC1-12	140	7.2	15.2	0.10	14.0	0.9	1.5
CC1-14	142	7.0	16.1	0.14	15.3	0.8	1.9
CC1-13	140	6.5	15.2	0.22	35.9	1.5	4.4
CC1-15	141	6.7	16.1	0.28	42.1	2.5	8.3
CC1-33	142	6.7	15.5	0.44	47.0	12.0	25.4
CC1-28	141	5.8	14.5	0.54	52.3	15.4	29.6
CC1-21	141	6.8	16.9	0.64	43.5	15.9	24.9
CC1-16	150	5.7	14.4	0.11	14.7	1.0	1.8
CC1-18	150	7.4	15.4	0.14	30.4	1.4	3.3
CC1-17	149	6.5	14.4	0.19	42.3	2.3	6.8
CC1-19	150	6.4	15.4	0.29	53.6	10.5	23.6
CC1-20R	149	6.9	15.4	0.37	50.6	15.8	30.3
CC1-27	154	6.4	14.5	0.54	54.6	18.1	32.1
CC1-22	148	7.1	16.9	0.64	44.0	15.4	24.9
CC1-32	166	8.2	15.4	0.20	49.2	2.6	7.8
CC1-31	167	7.2	15.5	0.30	46.1	13.7	27.5
CC1-25R	167	7.5	15.4	0.44	47.9	16.5	28.7
CC1-10	166	6.8	13.4	0.53	56.4	20.4	30.5
CC1-24	167	8.0	15.5	0.63	43.1	16.7	24.0

RUN NUMBER and REACTOR CONDITIONS	INITIAL SUGAR CONCENTRATION (g/L)			FINAL SUGAR CONCENTRATION (g/L)			EtOH (g/L)	FERMENTATION EFFICIENCY	
	glucose	xylose	TOTAL	glucose	xylose	TOTAL		Total	of xylose*
CC1-2 157°C, 3.6 min. 0.15% HCl	13.2	58.1	121.5	0	2.4	48.3	19.3	0.26	0.19
CC1-20R 149°C, 6.9 min. 0.37% HCl	29.3	50.1	93.6	0	4.5	9.8	10.3	0.12	0
CC1-21 141°C, 6.8 min. 0.64% HCl	31.8	44.0	87.1	0	4.5	11.0	13.4	0.18	0
CC1-22 148°C, 7.1 min. 0.64% HCl	32.2	45.8	92.2	0	20.0	32.0	18.9	0.31	0.05
CC1-25R 167°C, 7.5 min. 0.44% HCl	32.6	50.8	94.7	0	3.0	7.1	8.7	0.10	0
CC1-27 154°C, 6.4 min. 0.54% HCl	31.0	49.0	93.6	0	13.7	24.5	17.5	0.25	0.01
CC1-28 141°C, 5.8 min. 0.54% HCl	25.5	43.4	86.6	0	13.2	24.2	16.5	0.26	0
PCC1-4 150°C, 7.1 min. 0.57% HCl	8.2	44.4	60.9	0	3.1	8.9	8.9	0.17	0.08

* assuming C_6 fermentation is 100% efficient (ie: 0.51 g EtOH/g glucose) and no DP2+ sugars are oligomers of xylose.

TABLE 9 : ALPHA FLOC HYDROLYSIS (Pure Cellulose)

non volatile at ~15" Hg vac and ~60-70°C

DRY SOLIDS LEVEL and RUN NUMBER	V (ft/s)	TEMPERATURE PROFILE (°C)	ACID STRENGTH (%) (pH)	TOTAL YIELD OF NON-VOLATILES (%) +	YIELD OF GLUCOSE (%)
AF 2-1a (6.8%)	1.78	40' (0.37 min) 206.9 80' (0.75 min) 217.4 Boiler: 220	0.146 (1.60)	67.6	23.5
AF 2-1b (6.8%)	0.86	40' (0.78 min) 214.6 80' (1.55 min) 217.8 Boiler: 220	0.146 (1.60)	53.6	26.7
AF 2-2a (6.5%)	1.58	40' (0.42 min) 226.5 80' (0.84 min) 236.2 Boiler: 240	0.145 (1.63)	39.6	41.5
AF 2-2b (6.5%)	0.86	40' (0.78 min) 233.4 80' (1.55 min) 237.7 Boiler: 240	0.145 (1.63)	25.8	44.6
AF 2-3a (7.5%)	>0.81	40' : 247.8 80' : (< 1.6 min) 251.6 Boiler: 254	0.147 (1.75)	32.4	5.3
AF 2-3b (7.5%)	0.81	40' (0.82 min) 249.2 80' (1.65 min) 250.6 Boiler: 254	0.147 (1.75)	23.5	3.9
AF 2-4 (7.7%)	1.19	40' (0.56 min) 207.6 80' (1.12 min) 216.2 120' (1.68 min) 216.3 Boiler: 218	0.150 (1.59)	61.5	25.3
AF 2-5 (7.7%)	1.25	40' (0.53 min) 173.1 80' (1.07 min) 229.5 120' (1.60 min) 238.1 Boiler: 239	0.150 (1.59)	40.6	24.8
AF 2-6a (8.9%)	1.46	40' (0.46 min) 233 Boiler: 249	0.148 (1.53)	64.7	26.7
AF 2-6b (8.9%)	0.92	40' (0.72 min) 244.9 Boiler: 253	0.148 (1.53)	50.3	37.9

FIGURE 1: Process Flowsheet

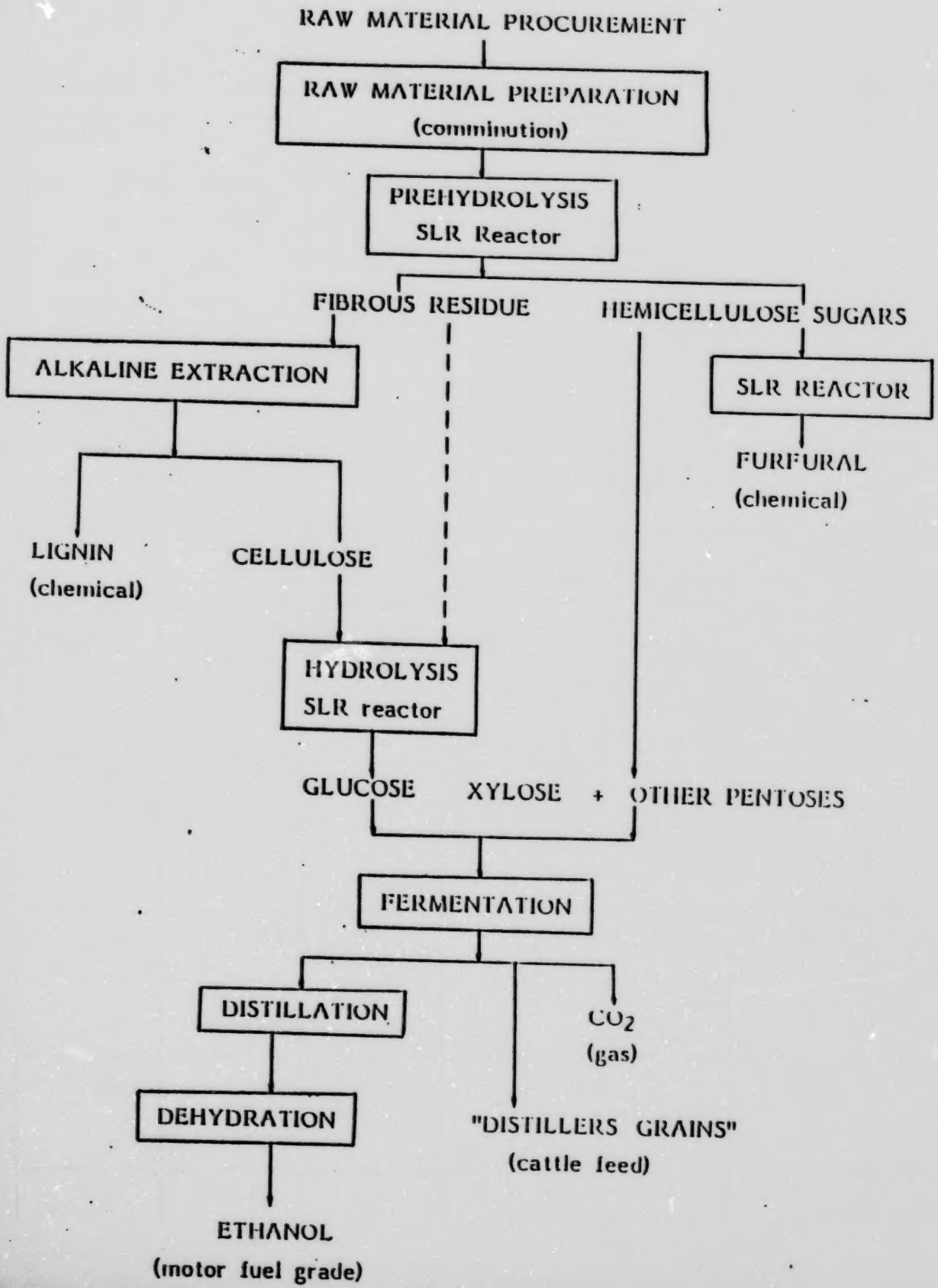


FIGURE 2 — Corn Cobs

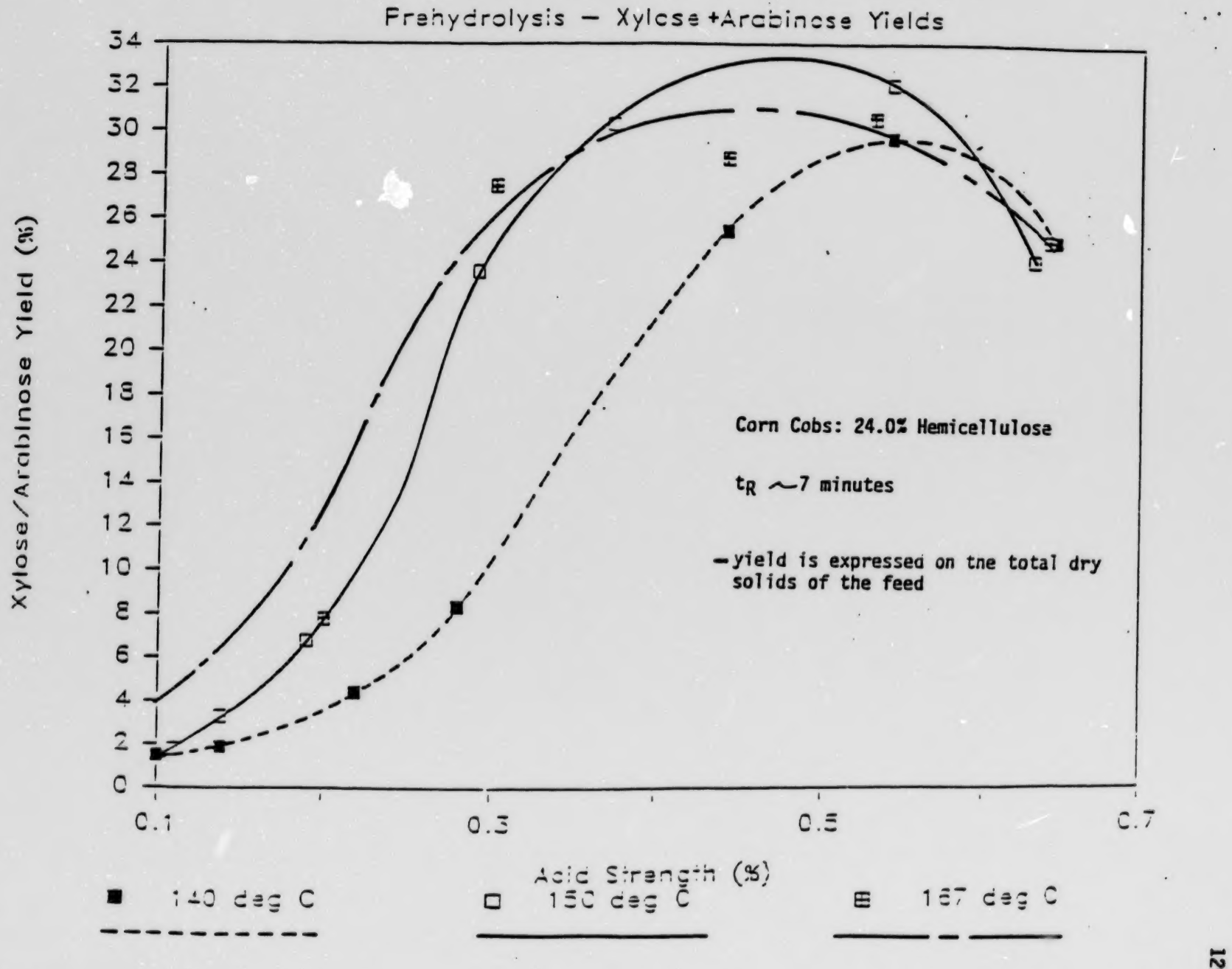


FIGURE 3 — Corn Cobs

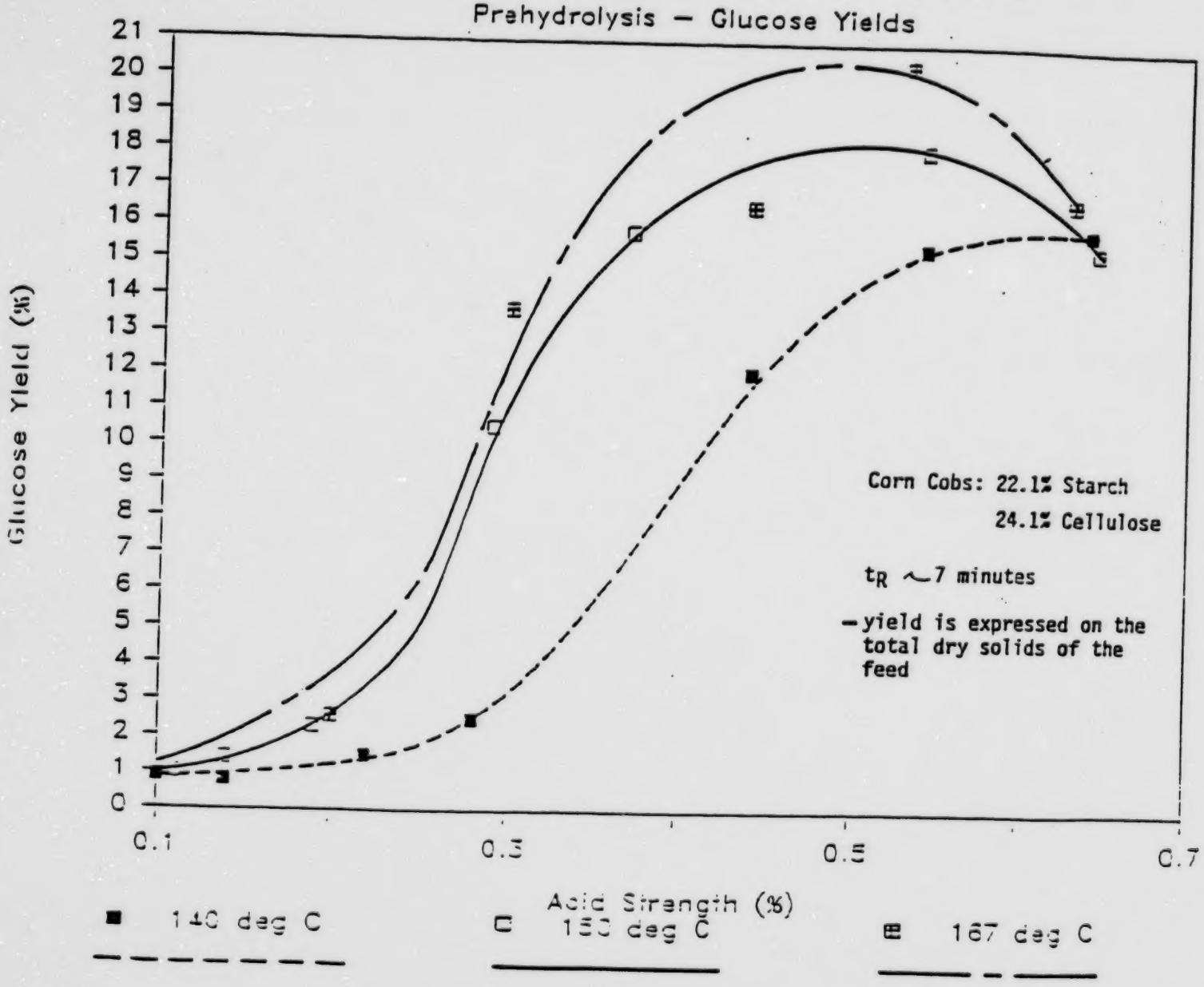
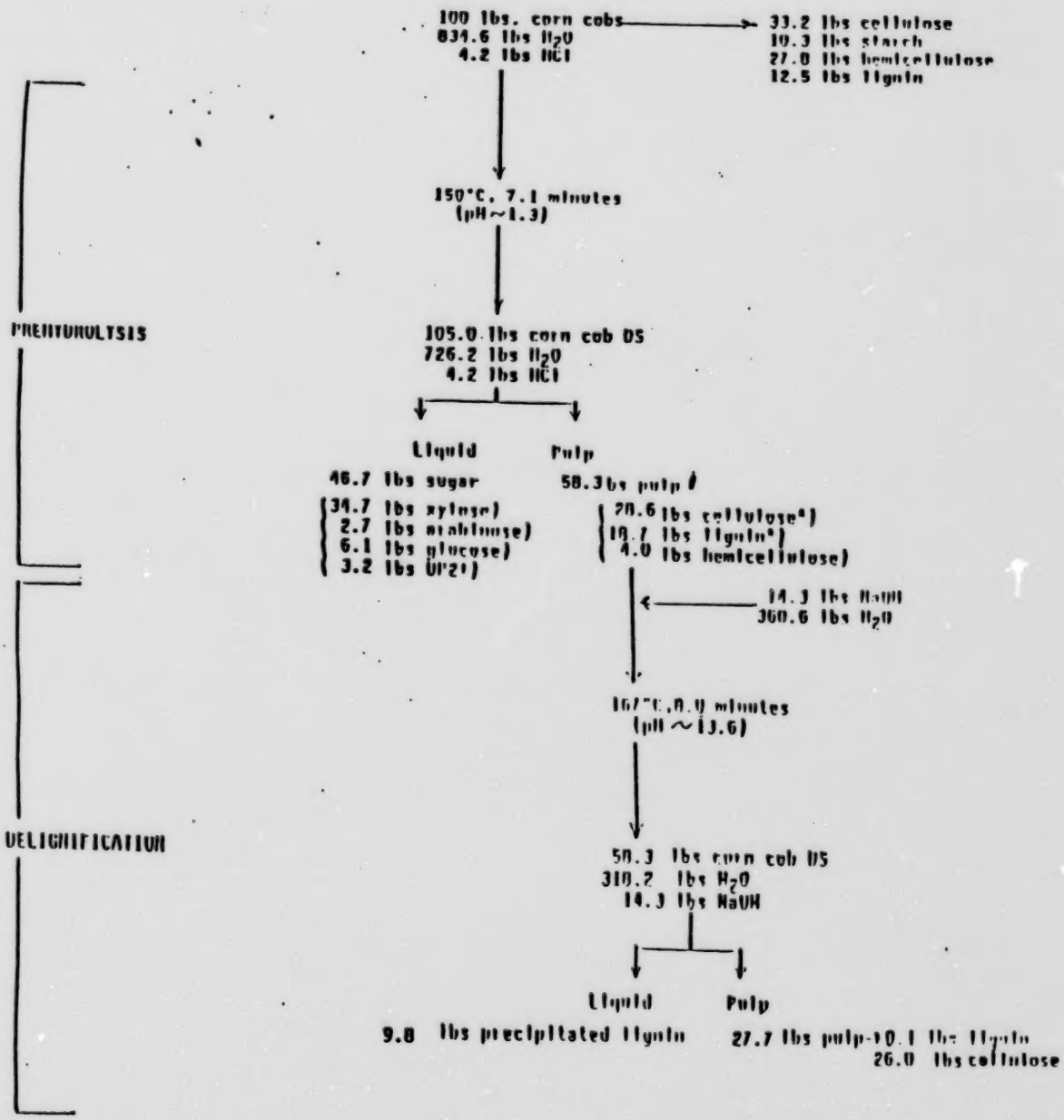


FIGURE 4: Prehydrolysis and Delignification of Corn Cobs: Mass Balance



- recovery of hemicellulose: i) based on sugars recovered = (34.7+2.7)/(1.136) (27.0) = 110%
  ii) based on residual pulp analysis = (27.0-4.0)/27.0 = 86%
- recovery of lignin based on original lignin in = 9.8/12.5
  = 78%
- yield of cellulose based on original cellulose in = 26.0/33.2
  = 78%

/ This value obtained by difference. The actual recovered weight of pulp was 49.7 lbs. The loss of 8.6 lbs was assumed to come entirely from the pulp fraction and therefore has been added on.

* It appears as if after the first stage some cellulose condenses onto the lignin and therefore analysis gives high lignin figures and low cellulose yields.

FIGURE 5A: Candida shehatae

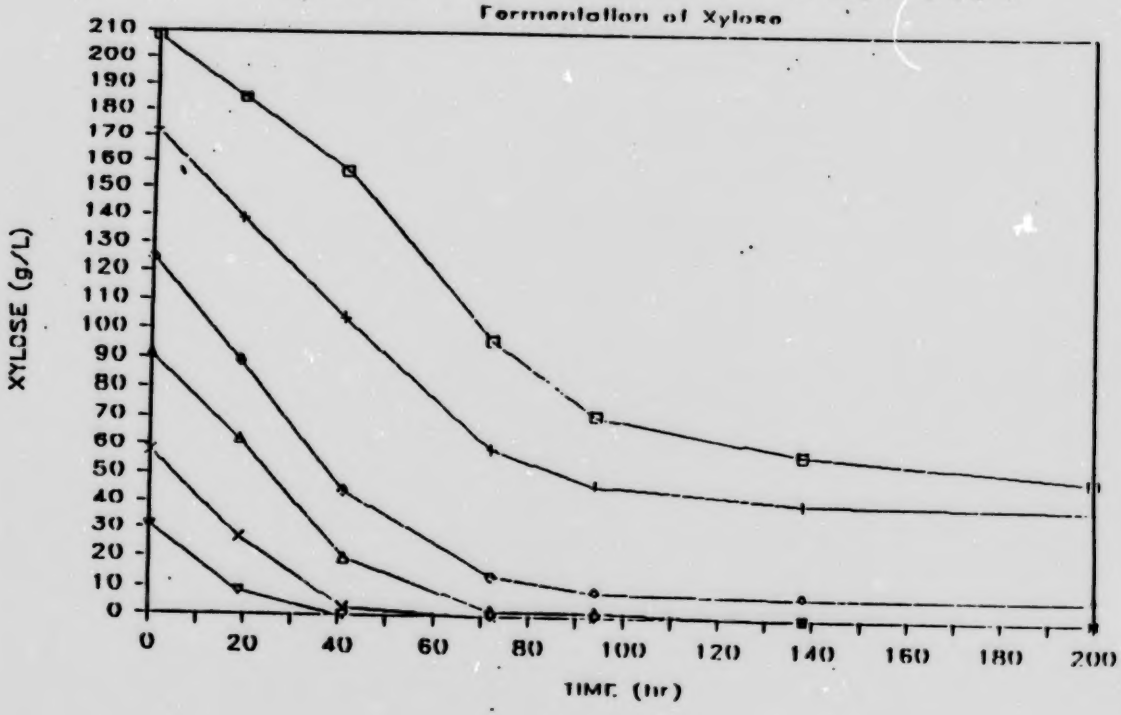


FIGURE 5B: Candida shehatae

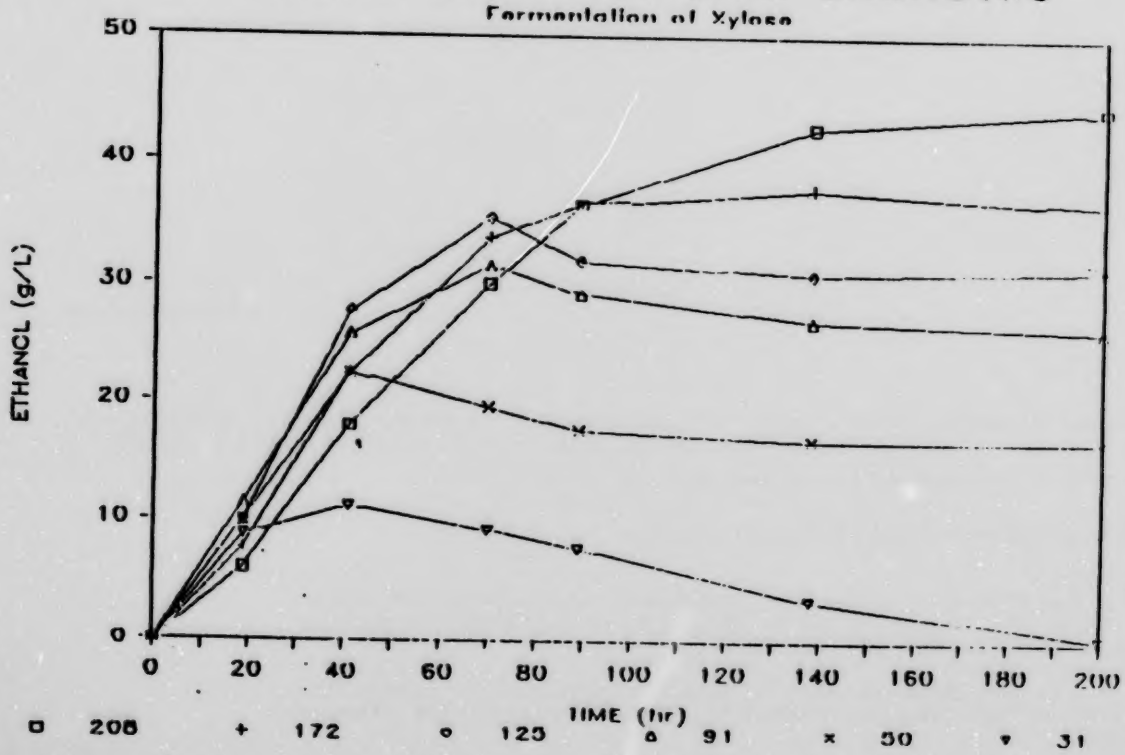


FIGURE 6A: Candida shehatae

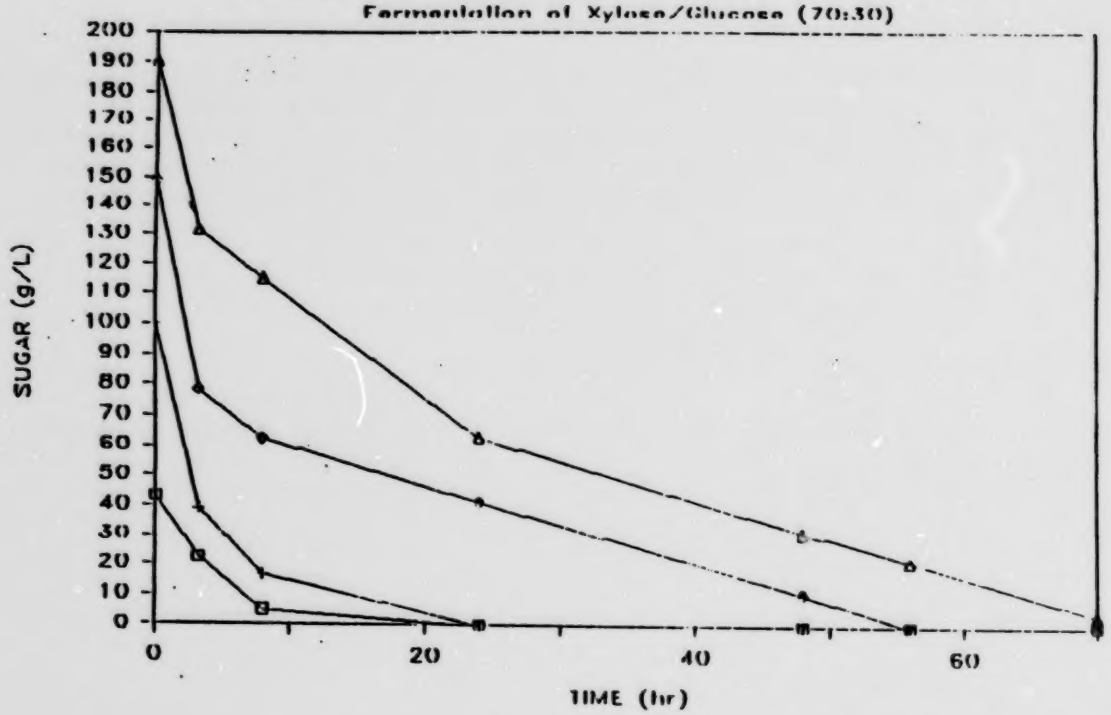
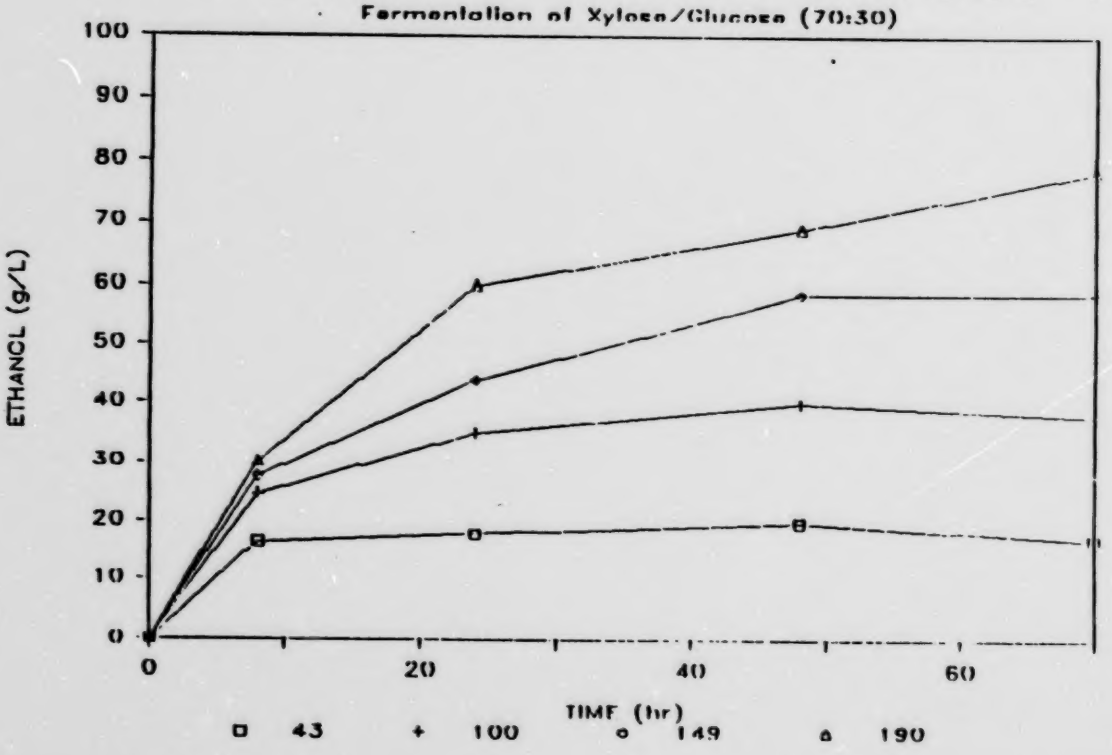


FIGURE 6B: Candida shehatae



BUTANOL PRODUCTION BY EXTRACTIVE FERMENTATION USING FLUOROCARBONS

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ABSTRACT

This paper describes a new system for producing butanol by extractive fermentation. Butanol has taken on new importance as an alcohol fuel by its use as cosolvent in methanol-gasoline motor fuels. While sugar or starch raw materials may be used as substrates, the butanol-forming organism is particularly interesting in its ability to ferment pentoses, enabling good yields of butanol from hydrolysates of pentose-rich feedstocks such as agricultural crop residues, bagasse and deciduous woods such as poplar and willow. The new process is designed to overcome product inhibition, a major handicap to commercial exploitation of the conventional butanol-acetone-ethanol process. Butanol concentrations over 1.5 to 2% have inhibited fermentation. In the process to be reported, butanol is extracted from the beer by fluorocarbons, especially "Freon 11", which enables butanol production to proceed. Fluorocarbons are particularly attractive extractants because of very low heats of vaporization, enabling the recovery of both extractant and product at low cost. This process is suitable for conventional batch fermentation; and other process models will be presented, including continuous tower fermentations with immobilized cells, and two-stage fermentations with butyric acid formers in the first stage and butanol formers in the second stage.

1. INTRODUCTION

Butanol has taken on new importance as an alcohol motor fuel because it is an effective cosolvent for methanol in gasoline. The use of methanol in gasoline is the subject of extensive trials in North America and in Europe, in part because of overcapacity of methanol production from natural gas in North America, and because of the prospect of Soviet natural gas deliveries in Western Europe which may also be converted to fuel

methanol. In the North American trials of methanol motor fuel, substantial proportions of butanol are being added as cosolvent, up to 50%. The butanol used in these trials is derived from petrochemical processes, and most of it is tertiary butanol, with some secondary butanol also being used. Present experience suggests that any of the butanols, primary, secondary or tertiary, are more or less equally effective as cosolvent for methanol in gasoline.

The orientation of our work is to provide butanol on a renewable resource base, since petrochemical butanol is limited. Butanol is a product of fermentation by bacteria, and fermentation butanol has been produced commercially in North America, but mainly under wartime conditions. The first commercial North American fermentation butanol plant was established in Toronto during World War I using the Weizmann strain of Clostridium acetobutylicum, with corn as substrate. The purpose of that industrial operation was to make the coproduct acetone, needed as a solvent in the manufacture of smokeless powder. Butanol later became important as a solvent for surface coatings, particularly motor vehicle paint. With the rapid growth of the petrochemical industry after the war, most acetone-butanol fermentation plants were shut down as uncompetitive with the petroleum sources. Presently the only substantial plant in operation is at National Chemical Products in South Africa, which is based on molasses as substrate. The South African process and plant have been described by Spivey (1).

A recent review by Volesky et al (2) provides somewhat more of the history and background of the process. Volesky lists the clostridia and bacilli reported as solvogenic in the literature. These were grown mainly on molasses, with or without conversion, with added protein or ammonia and calcium carbonate. Cl. acetobutylicum is capable of growth and solvent production on mashes of

various grain (corn, wheat, barley, rice) and tubers (potatoes, cassava, beets). He also mentions waste sulphite liquor as substrate. Mes-Hartree and Saddler (3) and Ounine et al (4) describe butanol production from the sugars which are common to hydrolysates of lignocellulosics such as wood. Arlie et al (5) of Institute Francais du Petrole (IFP) have described butanol formation using hydrolysates of wheat straw as substrate. Current reports are the IFP has under construction a demonstration scale fermentation butanol plant. These papers suggest that an alcohol fuel based on butanol fermentation of renewable resources is technically feasible.

A major handicap to commercial success of butanol fermentation is a severely limiting product inhibition. Butanol concentrations over 2.0% are significantly inhibitory, resulting in low concentrations in the beer and high distillation costs. The inhibition is not clearly understood, and may eventually be overcome by the development of resistant strains, using classical or genetic engineering techniques. Meanwhile, fermentation produces only quite dilute butanol beers.

The concepts on which the present research is based are designed to overcome the problem of product inhibition, to reduce considerably the cost of recovering butanol from the beer, and generally to provide a process with much improved technology and economics to enable fermentation butanol to become once again a commercial product at a cost which makes its use as motor fuel viable. The new process element in our configuration is Levy's 1981 patent (6) describing extraction of alcohols from water solutions with fluorocarbon extractants. These extractants, sold under the trade name "Freons", have extremely low latent heats of vaporization and as such are widely used in household and other refrigeration applications. The low heat of vaporization results in low energy, low cost distillation. As extractants, fluorocarbons carry the promise of recovering butanol by extraction to be followed by low cost distillation to recover the Freon for reuse as extractant and the butanol as the desired product. Accordingly laboratory experiments have been carried out to determine the extent of these possibilities.

2. FERMENTATION

This work was carried out at the University of Vermont, Burlington. Batch and continuous fermentations were run using two different cultures, Cl. acetobutylicum ATCC 824, and Bacillus butacone isolated in the Toronto laboratory from humic soil in accordance with the procedure of Hall (7). Both cultures were effective butanol producers. The culture medium contained 2% bactopeptone (Difco), 1% yeast extract (Difco) and 6% glucose. Fermentation was anaerobic in a nitrogen atmosphere at 37°C. Solvent concentrations were determined by a Perkin Elmer 900 GC with a flame ionization detector, using 6% FFAP on Poropak Q (80/100 mesh). A typical fermentation produced a beer containing butanol 2.0% (weight/volume), acetone 1.2% and ethanol 0.4%, for a total solvent concentration of 4.0%. The yield of solvent was 0.30 to 0.32 g/g glucose. The same yield was obtained in the continuous chemostat and the batch runs.

3. FLUOROCARBON EXTRACTION

The fermentation beer was extracted with Freon 11, which is trichlorofluoromethane. Freon is a registered trade mark of duPont. The Freon 11 used in these experiments was refrigeration grade. The partition coefficients for butanol, acetone and ethanol were determined using a beer containing 1.18% butanol, 0.52% acetone and 0.16% ethanol. The partition coefficients (concentration in extract/concentration in aqueous phase at equilibrium) was 0.40 for butanol, 0.44 for acetone, while ethanol was insoluble in Freon 11. Butyrate and acetate also remained in the beer, and were not extracted by Freon 11. The presence or absence of the cells in the beer did not affect the partition coefficients. Ten successive extractions with the Freon removed 83% of the butanol and 100% of the acetone.

Because of its 24° boiling point at atmospheric pressure, the Levy continuous extraction process is carried out at essentially room temperature. The fluorocarbons are evaporated using reduced pressure on the system and are recondensed by applying pressure. The condensing vapors are brought through a heat exchanger where the heat of condensation is used to evaporate the extractant fluorocarbon from the extract, so that there is regenerative heat recovery. The energy input to the system is the shaft

work applied to the compressor. This is essentially the same as a refrigeration or heat pump cycle.

The flow sheet for the process is shown in Figure 1. The equipment includes an extractor operating under positive pressure and equipped with a feed line for the fermentation beer, and a raffinate line for the extracted beer, as well as connections to the extractant system. The extractant system has a compressor loop which draws the fluorocarbon vapors off the extract solution to provide product, and recompresses the fluorocarbon vapors to liquid to return to the extractor. A heat exchanger transmits the heat from the condensing extractant vapors to the evaporating extractant.

Freon 11 has heat of vaporization 0.18 Mj/kg, specific heat 0.209, density 1.464, boiling point 24°C.

Using the beer composition and partition coefficients given above, calculations show energy requirement in the product recovery system to be 5500 BTU/gallon solvents, and an energy efficiency of 83%. The product recovery system requires 1.61 kWh/gallon which, at $0.03/kWh, amounts to $0.048/gallon.

4. ECONOMICS

A plant to make 15 million gallons per year of n-butanol by this process will also make 7.5 million gallons per year of acetone, and 2.5 million gallons per year of ethanol, a total of 25 million gallons of solvent per year. Other products will include hydrogen, carbon dioxide, dried distillers' grains (DDG), and in the case of whey as raw material, protein. Such a plant is estimated to require $17 to $22 million investment, depending upon the feedstock. This is to be compared with Volesky's (2) estimate of $28.9 million investment for a conventional fermentation n-butanol plant of this size. Production costs, exclusive of feedstock, are estimated to be $6.6 million, including depreciation and plant overheads. Annual income for such an operation will be $24.4 million from the solvents and $14 to $17 million from the other products.

The only presently operating commercial fementation n-butanol plant, of conventional design (1), uses molasses as raw material. The price of molasses varies widely, from

$55/tonne on the U.S. West Coast to $110/tonne at Montreal. At 55 gallons of solvent per tonne of molasses, a plant of this size will require 450,000 tonnes/year. At a price $55/tonne, raw material costs will be $25 million. With income from the sale of solvents and the other products of $38.9 million, return on the investment of $17 million is estimated at 42.9%.

With whey as raw material, the return is even more favorable. With income, including sales of protein, of $42.6 million and production costs, including whey, of $14 million, return on investment of $22 million is 130%. A high return on investment of the order of 100% could be expected with sulphite waste liquor.

When corn is the raw material, this plant would require 9.5 million bushels/year, which, at $2.71/bushel, is a corn cost of $25.8 million. With income of $46.6 million, return on investment of $20.3 million would be 70.0%.

To use wood, corn cobs or other lignocellulosics as raw material, yields of 73 gallons solvent/tonne of corn cobs, or 66 gallons solvent/tonne pine sawmill residues may be expected (8), but investment of $28 million in a hydrolysis plant could be required, and addtional operating costs of $25.9 million to make the hydrolysate, including the cost of raw materials of $15/dry tonne, would be incurred. Return on total investment of $52 million would be 27.1%. The price of $15/tonne for corn cobs or sawmill residues is reasonable, even high in some situations. Plantation wood can be expected to be more expensive, and at $30/tonne, the ROI would be reduced to 17.3%.

Thus in preferred order of return on investment waste materials such as whey and sulphite waste liquor are preferred, while corn, molasses, corn cobs and sawmill residues also give quite acceptable return on investment.

5. PROCESS DESIGN DEVELOPMENT

Recent developments in fermentation technology for production of ethanol have included continuous fermentation using microbial cells immobilized on beads (9), film (10) and fibre (11). Haggstrom (12) has

demonstrated the successful production of butanol by continuous fermentation by immobilized cells of Cl. acetobutylicum ATCC 824 on glucose-butyric acid (10:1) substrate. Such a continuous fermentation system would lend itself readily to the extractive fermentation described in the present paper. Some of the newer immobilized cell systems are said to be highly productive on a reactor volume basis (10,11) and have the potential for reducing capital as well as operating costs.

It is generally accepted that in the butanol fermentation process butyric acid is formed before butanol. Haggstrom and Molin (13) have shown much increased butanol production when butyric acid is added to the medium. Butyric acid may also be made by fermentation, as reviewed in some detail by Playne (14). It has also been shown that propionic acid can be made continuously in towers by bacterial cells immobilized on small lumps of limestone (15) and it would be expected that butyric acid can also be produced by immobilized bacteria in continuous fermentation systems. Butyric acid so produced may be an additional feed to a butanol production system.

A flow sheet for a butanol fermentation process incorporating these elements is shown in Figure 2. It is a 2-stage continuous fermentation, using Cl. butyricum in the first stage to produce butyric acid, which becomes part of the substrate for further fermentation by Cl. acetobutylicum, or an equivalent butanol former. In both stages of fermentation, immobilized cells are used. The product is extracted by a fluorocarbon as described.

6. CONCLUSIONS

While many of the individual elements of the system described in the preceding paragraph have been studied in the laboratory, the total system has yet to be assembled. This research is being carried further. Such a butanol production system should prove to have commercial potential. As pointed out above, commercial success will depend upon utilization of a cheap raw material as source of substrate. If successful, this will be an alcohol fuel based upon renewable resources.

REFERENCES

1. M.J. Spivey, Process Biochemistry, November 1978, p2 - 4, 25.

2. B. Volesky, A. Mulchandani and J. Williams, Ann. N.Y. Acad. Sciences, 369, Biochem. Eng. 2, p205 - 218 (1981).

3. M. Mes-Hartree and J.N. Saddler, Biotech. Lett. 4, No. 4 p247 - 252 (1982).

4. K. Ounine, H. Petitdemage, G. Ravel and R. Gay, Biotech. Lett. 5, No. 9, p605 - 610 (1983).

5. J.P. Arlie, J.P. Vandecasteele and P. Leprince, Proceedings OECD Workshop No. 2, 8 - 10 October, 1980 p336 - 358.

6. S. Levy, U.S. Patent 4,260,836, Apr. 7, 1981.

7. H.E. Hall, U.S. Patent 2,147,487, Feb. 14, 1939.

8. M. Wayman, A. Tallevi and B. Winsborrow, Hydrolysis of Cellulosics to Fermentable Sugars by Sulphur Dioxide, This Symposium.

9. G. Durand and J.M. Navarro, Process Biochemistry, September 1978, p14 - 23.

10. S. Nojima, Chem. Econ. and Eng. Review 15, No. 4, p17 - 22 (1983).

11. R.A. Clyde, U.S. Patent 4,407,954, Oct. 4, 1983.

12. L. Häggström, Advances in Biotechnology Vol II, Pergamon 1981, being Proceedings of the Sixth Intern. Ferment. Symp., London, Canada July 20 - 25, 1980, p79 - 83.

13. L. Häggström and N. Molin, Biotech. Lett. 2 No. 5, p241 - 246 (1980).

14. M.J. Playne, In "Comprehensive Biotechnology" Part 3, Vol. 2, Chapter 13.4, Pergamon, 1983.

15. M.E. Martin, M. Wayman and G. Graf, Can. J. Microbiol. 7, p341 (1961); U.S. Patent 3,067,104, Dec. 4, 1962.

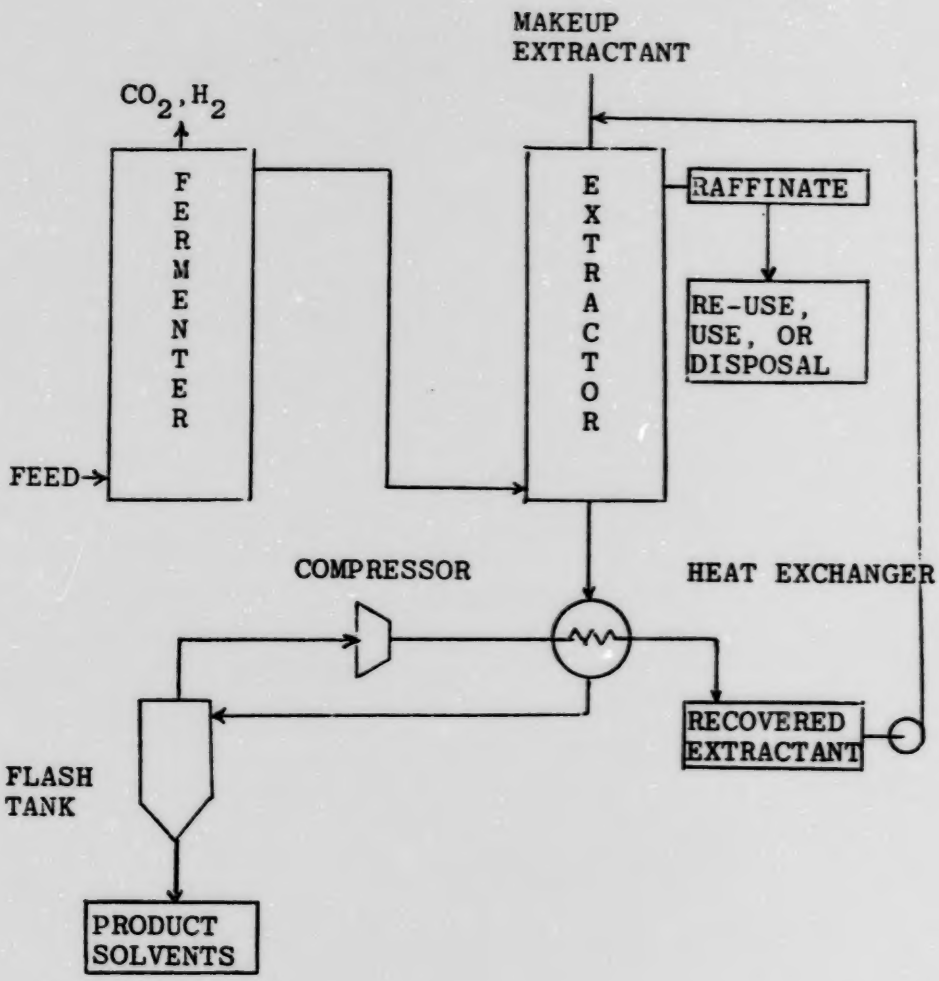

FIGURE 1. EXTRACTIVE FERMENTATION

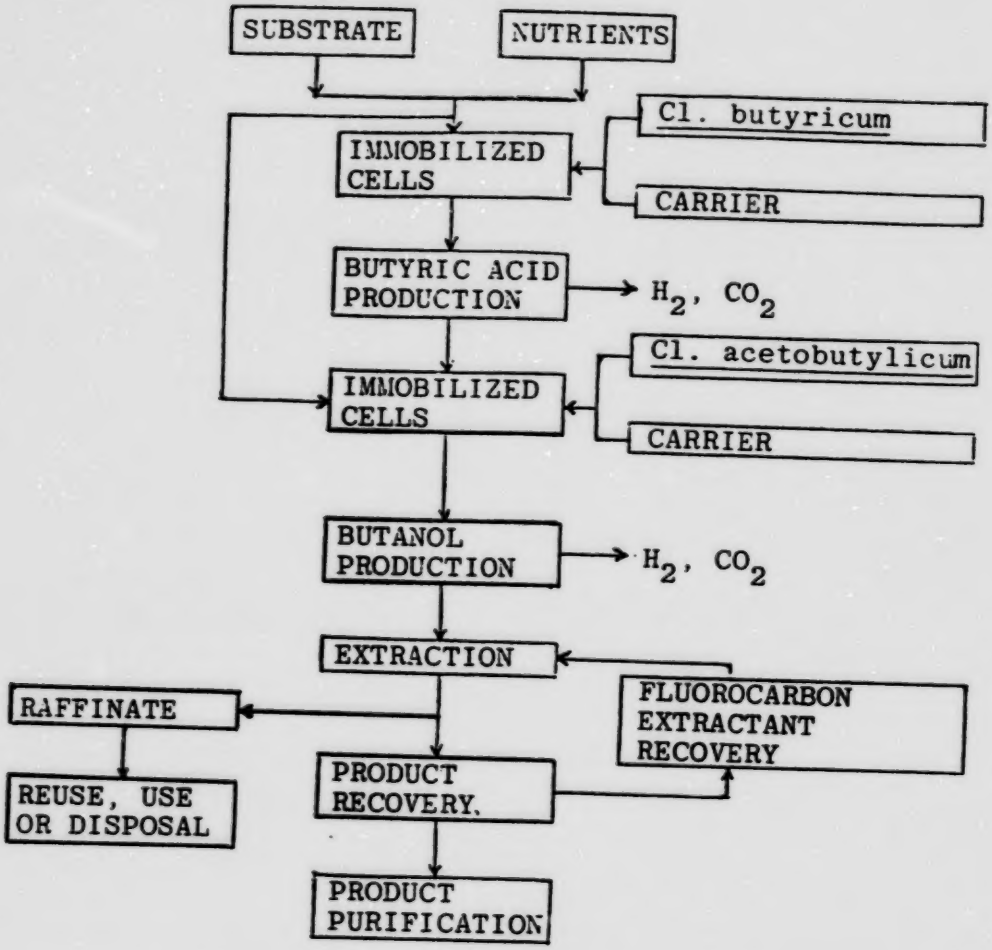

FIGURE 2. SCHEMATIC FLOW DIAGRAM OF BUTANOL FERMENTATION SYSTEM

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ACETONE-BUTANOL FERMENTATION OF XYLOSE AND SUGAR MIXTURES

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SUMMARY

Three strains of Cl. acetobutylicum and one of Cl. butyricum have been tested for their ability to ferment xylose to butanol. ATCC 824 and NRRL 527 produced 0.28 g solvents/g xylose, while ATCC 8260 and NRRL 594 produced much butyric acid. In 2-stage fermentations in which ATCC 8260 or NRRL 594 acted upon xylose for 12 to 20 h, followed by NRRL 527 for a total of 3 days, yields of solvent were better, 0.32 g/g xylose. Upon fermenting a mixture of sugars simulating sulphite waste liquor 0.36 g solvents/g sugar were obtained. Sugar consumption in both cases was about 96%.

INTRODUCTION

Interest in acetone-butanol fermentation has attracted renewed attention (Spivey, 1978; Jones, 1982; Long, 1983; Wayman 1984). This interest arises from increasing costs of butanol from petrochemical sources, and from a new market for butanol as cosolvent in methanol-gasoline motor fuel. The traditional substrates for acetone-butanol fermentation are corn steep liquor and molasses. Attention has also turned to wood hydrolysates and sugar mixtures simulating wood hydrolysates (Ounine, 1984; Yu, 1984). Our interest in sulphite waste liquor (Wiley, 1941) arises from present processes for production of vanillin, which include the step of de-sugaring the sulphite waste liquor prior to vanillin production by fermentation to ethanol by ordinary bakers' yeast (Craig, 1973). Following distillation of the ethanol, the lignin and residual sugars in solution are processed by catalysed alkaline air oxidation to vanillin. Since the wood-derived sulphite waste liquor contains, in addition to glucose, also xylose and arabinose, which are scarcely fermented by the yeast, these pentoses are present during the oxidation, and contribute materially to vanillin production costs. Our general concept is that acetone-butanol fermentation, which will also ferment the pentoses, will perform a more complete de-sugaring of the waste sulphite liquor, thereby reducing the cost of vanillin production. This paper describes experiments

carried out with the pure sugars glucose, xylose, mixtures of glucose and xylose, and a sugar mixture made up to simulate sulphite waste liquor.

MATERIALS AND METHODS:

Cultures: Clostridium butyricum ATCC 8260 (University of Wisconsin 39). Cl. acetobutylicum ATCC 824, NRRL 527, NRRL 594. The lyophilized culture was germinated in reinforced Clostridium medium (RCM; Long, 1983), followed by sporulation in 10% corn meal mash at 35°C for 7 days. The spores were collected by centrifugation, suspended in distilled water and stored at 4°C. For inoculation, the spore suspension was heat shocked at 80°C for 5 min, transferred to corn-liver medium (6% corn mash, 0.5% Bacto-liver; CL) and incubated at 35°C for 24 h in a reducing atmosphere in an anaerobic glove box (Santangelo, 1984). All fermentations were carried out in the anaerobic atmosphere at 35°C for 2 or 3 days

Media: In this report, all initial sugar concentrations were 30 g/ℓ. A 5-sugar mixture simulating commercial coniferous sulphite waste liquor (The Ontario Paper Company, Thorold, Ontario) contained, in g/ℓ, mannose 12, xylose 6, galactose 5.5, glucose 4 and arabinose 2.5. In the glucose-xylose experiments, Monot's nutrients (Monot, 1982) was used. In the experiments with the 5-sugar mixtures, in addition to Monot media, other media were (i) diammonium phosphate 5 g/ℓ + corn steep liquor 50 g/ℓ (DAP-CSL); (ii) DAP 5 g/ℓ + molasses 10 g/ℓ (DAP-ML); (iii) DAP 5 g/ℓ + malt extract 10 g/ℓ (DAP-MA); (iv) Peptone 20 g/ℓ + yeast extract 10 g/ℓ (PY); and (v) DAP 5g/ℓ + CL 50 g/ℓ (DAP-CL). Starting pH was 6.4 in all cases. Inoculum for single stage fermentation was 10% by volume (about 0.2 g/ℓ) of 24 h cultures growing in CL. For 2-stage fermentations 5% by volume was added at each stage, after 12, 16 or 20 h.

Analysis: solvents and acids by GC; sugars by DNS (Miller, 1959).

RESULTS

Results of fermentations of xylose, glucose and their mixtures are shown in Table 1, 2-stage fermentation of xylose in Table 2, and fermentation of the 5-sugar mixture in Table 3.

DISCUSSION

The results in Table 1 show that all 4 cultures produce solvents from glucose and glucose 80%-xylose 20% in acceptable yield (0.25 to 0.35 g solvents/g sugar consumed). While the best yield was obtained with NRRL 594 on glucose medium, that organism as well as ATCC 8260 (39) produced much less solvent when fermenting xylose or glucose 20%-xylose 80%, forming butyric acid instead, while ATCC 824 and NRRL 527 produced more solvent fermenting xylose than glucose. In other experiments, to be reported, we found that ATCC 824 and NRRL 527 are not adaptable to sulphite waste liquor, while ATCC 8260 (39) and NRRL 594 are more readily adaptable to sulphite waste liquor.

The objective of the 2-stage fermentations was to start the butyric acid producers on xylose, then adding ATCC 824 or NRRL 527 to convert the butyric acid to solvents. This strategy failed with ATCC 824 but was successful with

NRRL 527, as shown in Table 2. NRRL 594 was found to be somewhat slower than ATCC 8260 (39), but in 3 days the yield of solvents was excellent and sugar consumption was 96% or better.

When ATCC 8260 (39) was used to ferment a mixture of sugars simulating sulphite waste liquor, the results shown in Table 3 were obtained. In this sugar mixture, the hexoses, principally mannose, were 71.7%. Media DAP-CSL gave the highest yield, 0.36 of solvents per g sugar consumed, along with the best sugar consumption. Monot's medium and PY were not quite as good. With DAP-ML sugar consumption was poor and in the case of a lower starting pH, 6.0, the pH fell to very low levels, 3.7, and much ethanol was formed. With DAP-MA or DAP-CL, solvent production was very poor.

We are now carrying out adaptation of these microorganisms to sulphite waste liquor and its fermentation to acetone-butanol, as a means of reducing sugar content prior to vanillin production.

ACKNOWLEDGEMENT

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LITERATURE CITED

Craig, D., 1973. Am. Inst. Chem. E. Symp. Ser. 69:1-5.

Jones, D.T., van der Westhuizen, A., Long, S., Reid, S.J. and Woods, D.R., 1982. Appl. Environ. Microbiol. 43:1434-1439.

Long, S., Jones, D.T. and Woods, D.R., 1983. Appl. Environ. Microbiol. 45:1389-1393.

Miller, G.L., 1959. Anal. Chem. 3:426.

Monot, F., Martin, M.R., Petitdemange, H. and Gay, R., 1982. Appl. Environ. Microbiol. 44:1318-1324.

Ounine, K., Petitdemange, H., Raval, G. and Gay, R., 1983. Biotech. Ltss. 5:605-610.

Santangelo, J.D., 1984. Master's thesis, The University of Vermont.

Spivey, M.J., 1978. Proc. Biochem. 13:2-5.

Wayman, M., Husted, G.R., Santangelo, J.D., and Levy, S., 1984. Proceedings VI Intern. Symp. Alcohol Fuels Techn. 2: 239-247.

Wiley, A.J., Johnson, M.J., McCoy, E. and Peterson, W.H., 1941. Ind. Eng. Chem. 33:60-6-610.

Yu, E.K.C., Deschatelets, L. and Saddler, J.N., 1984. Biotech. Letts. 327-332.

Table 1. Acetone-butanol fermentation of glucose and xylose; Monot's nutrients, sugar 30 g/ℓ, 3 days, 35°C, anaerobic, initial pH 6.4.

Culture	Substrate	Final pH	Sugar Used, %	Products, g/ℓ E	A	B	Total	Bua*	g solvent/ g sugar
ATCC 824	Glu	4.4	95.7	0.39	2.07	4.87	7.33	+	0.248
	Glu 80%- Xyl 20%	4.3	96.1	0.39	2.33	5.22	7.94	+	0.254
	Glu 20%- Xyl 80%	4.4	85.6	0.29	2.17	4.12	6.58	++	0.276
	Xyl	4.4	90.7	0.38	2.40	4.74	7.52	++	0.282
NRRL 527	Glu	4.4	95.9	0.50	2.57	5.02	8.09	+	0.273
	Glu 80%- Xyl 20%	4.4	96.0	0.57	2.49	5.38	8.44	+	0.284
	Glu 20%- Xyl 80%	4.5	93.0	0.46	2.56	5.23	8.25	+	0.313
	Xyl	4.5	96.1	0.51	2.74	4.92	8.17	+	0.289
ATCC 8260	Glu	4.8	95.8	0	3.29	5.51	8.80		0.297
	Glu 80%- Xyl 20%	4.6	89.3	0	2.23	6.05	8.28	+	0.300
	Glu 20%- Xyl 80%	4.6	-	0	1.03	4.44	5.47	++	-
	Xyl	4.6	-	0	low	low	low	++++	-
NRRL 594	Glu	5.0	91.5	0.25	2.75	7.06	10.06		0.356
	Glu 80%- Xyl 20%	4.9	88.1	0	1.82	5.70	7.52		0.276
	Glu 20%- Xyl 80%	4.7	81.2	0	1.09	4.69	5.78	+	0.251
	Xyl	4.5	-	0	low	low	low	++++*	-

* E = ethanol; A = acetone; B = n-butanol; Bua = butyric acid; ++++ = 3 to 5 g/ℓ.

Table 2. Two-stage acetone-butanol fermentation of xylose; medium 30 g/ℓ xylose + Monot's nutrients, pH 6.4.

First Stage Culture	Second Stage NRRL 527	Ferm. for days	pH	Sugar g/ℓ	Used %	Products (g/ℓ) E	A	B	Total Solv.	Bua*	g solvent/ g sugar
ATCC 8210 (39)	at 12 h	2	4.7	27.41	91.4	0.68	2.52	5.62	8.82	+	0.314
		3	4.75	28.87	96.2	0.80	2.04	6.25	9.09	–	
	16	2	4.8	19.37	64.6	0.39	1.30	4.16	5.85	+	0.307
		3	4.75	29.11	97.0	0.62	2.39	5.91	8.92	–	
	20	2	4.6	16.34	54.5	0	0.87	3.17	4.04	+	0.315
		3	4.8	29.05	96.8	0.75	2.40	6.03	9.17	–	
NRRL 594	12	2	4.5	15.04	50.1	0	1.50	2.82	4.32	+	0.242
		3	4.7	29.06	96.9	0.45	1.94	4.65	7.04	–	
	16	2	4.4	12.08	36.7	0.32	0.88	2.44	3.64	+	0.319
		3	4.75	30.33	92.2	0.58	2.61	6.49	9.68	–	
	20	2	4.7	17.08	56.9	0.30	1.58	3.75	5.63	+	0.332
		3	4.7	29.00	96.7	0.86	2.48	6.28	9.62	–	

* E = ethanol; A = acetone; B = n-butanol; Bua = butyric acid.

Table 3. Acetone-butanol fermentation of 5-sugar solution simulating sulphite waste liquor, total sugars 30 g/ℓ, by ATCCe8260 (39), 35°C, 70 h.

Media	pH		Sugar (g/ℓ)			Products (g/ℓ)				g solvent/ g sugar
	original	after ferm.	original	used	% used	E	A	B*	Total	
DAP-CSL	6.5	5.3	28.49	27.51	96.6	0.33	2.60	7.07	10.00	0.364
	6.0	5.0	28.49	27.44	96.3	0.22	2.70	6.75	9.67	0.352
Monot's	6.5	4.5	30.41	29.14	95.8	0.21	2.59	6.00	8.80	0.302
	6.0	4.4	30.41	28.76	94.6	0.20	2.97	5.63	8.80	0.306
PY	6.5	4.95	28.42	16.29	57.3	0.18	1.15	3.39	4.72	0.290
	6.0	5.2	28.42	21.40	75.3	0.19	1.86	4.74	6.79	0.317
DAP-ML	6.5	4.6	30.67	18.41	60.0	0.20	2.50	4.21	6.91	0.375
	6.0	3.7	30.67	17.53	57.2	4.26	1.33	1.86	7.45	0.425
DAP-MA	6.5	4.95	36.57	19.25	54.0	-	-	-	<5	-
	6.0	4.6	36.57	-	-	-	-	-	<2.5	-
DAP-CL	6.5	4.8	27.07	12.03	44.4	-	-	-	<3	-
	6.0	4.6	27.07	5.83	21.5	-	-	-	<1.5	-

* E = ethanol; A = acetone; B = n-butanol.

PRODUCTION of ACETONE-BUTANOL by EXTRACTIVE FERMENTATION using DIBUTYLPHTHALATE as EXTRACTANT

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ABSTRACT

Extractive fermentation of glucose, glucose-xylose mixtures and hydrolysates of lignocellulosics to acetone-butanol solvents were studied and compared with similar fermentations in the absence of extractant. The extractant selected for this research was dibutylphthalate which, in addition to having satisfactory physical properties for this purpose, is non-toxic and mildly stimulating to the growth of the organism used, Clostridium acetobutylicum P262. Sugar concentrations mainly in the range of 80 to 100 g/l resulted in solvent concentrations of 28 to 30 g/l in 24 h extractive fermentations, compared to 18 to 20 g/l for non-extractive control fermentations. Conversion factors of 0.33 to 0.37 g solvents/g sugar consumed were obtained. Rapid fermentation was achieved by high cell concentrations and cell recycle from each 24 h fermentation to the succeeding similar 24 h fermentation. Somewhat higher nutrients were also helpful. By this means, 255 l of acetone-butanol solvents were obtained per tonne of aspen wood, 298 l per tonne of pine and 283 l per tonne of corn stover. Such high product yields from inexpensive substrates offer the prospect of economic viability for the process.

INTRODUCTION

The phase-out of lead in gasoline as an octane booster has provided new markets for alcohol-gasoline blends for motor fuel. The least expensive alcohol available for this purpose is methanol. However, to be blended into gasoline methanol requires a co-solvent, and butanol, which has been added to gasoline for many years, appears to be the co-solvent of choice. Fermentation butanol was well established as an industrial product in the early part of this century[1] but in the past few decades butanol has been obtained as a product of petrochemical operations. With the expectation of a long-term shortage of inexpensive petroleum supplies, interest in acetone-butanol fermentation has increased[2,3]. Acetone-butanol fermentation incurs high production costs because the organisms used Clostridium acetobutylicum, Cl. butyricum, exhibit strong product inhibition, resulting in low concentrations of butanol, acetone and ethanol, up to 2.0 to 2.5% of total solvents, in the beer. Product distillation from these dilute solutions consumes much energy. While product inhibition causing high recovery costs is common to many industrial fermentations, the need for an alternative is especially compelling for a low-priced product such as a motor fuel co-solvent. A review of other process designs for the production of fermentation ethanol concluded that extractive fermentation offers the prospect of immediate energy and other economic benefits[4]. The feasibility of utilizing liquid-liquid extraction to overcome product

inhibition during ethanol fermentation[5,6] and butanol fermentation[7-9] has been discussed, with encouraging conclusions.

The present report describes an investigation into extractive acetone-butanol fermentation, and in particular indicates the potential of dibutylphthalate as extractant during fermentation[10], to obtain higher solvent concentrations and good solvent yields. Here the product is removed from the broth during the fermentation by in situ liquid liquid-extraction. Pure sugars and also hydrolysates of lignocellulosic feedstocks were used as substrates. The objective of this work is to provide data and suggest a process with improved technology and economics to enable fermentation butanol to become once again an industrial product at a cost which makes its use as a motor fuel co-solvent feasible.

MATERIALS AND METHODS

Reagents

The extractant employed in this research was dibutylphthalate (Baker ≥ 99%).

Chemicals for medium preparation were as follows, per liter of distilled water: 2.2 g ammonium acetate (Fisher); 0.5 g KH_2PO_4 (BDH); 0.5 g $K_2HPO_4.3H_2O$ (BDH); 0.01 g NaCl (BDH); 0.2 g $MgSO_4.7H_2O$ (MCB), 0.01 g $FeSO_4.7H_2O$ (Baker); 0.01 g $MnSO_4.4H_2O$ (BDH); 1.0 mg p-aminobenzoic acid (Nutritional Biochemical Corporation); 0.01 mg biotin (Baker).

Enzymes used were cellulase (Meicelase, Meiji Seika), hemicellulase (Miles), cellobiase (Novo) pectinase (Rohm and Haas) and glucoamylase (Novo). The enzyme mixtures used contained as "promoters " Vitamin B_{12} (Wampole) and trace elements[11].

Organism and Culture Maintenance

Clostridium acetobutylicum P262, a strain which ferments both hexoses and pentoses efficiently to acetone-butanol, was obtained from D.R. Woods (Department of Microbiology, University of Capetown, Rondebosch 7700, South Africa). The culture was maintained and sporulated in sporulation minimal medium[12].

Preparation of Inoculum

10 ml of spore suspension was heat shocked at 80°C for 5 min and cooled rapidly to room temperature in an ice bath. The spore suspension was then inoculated (10% by volume) into corn-liver medium[3]. After 24 h, the non-vegetative cell suspension was inoculated (10% by volume) into medium containing Monot's nutrients[13] and 80 g/l glucose. After 24 h incubation at 35°C on a reciprocating shaker in an anaerobic glove chamber[14], the cells were centrifuged and washed with saline. The resultant pellet was resuspended in medium and inoculated into 250 ml fermentation flasks. Cell concentrations were high, as indicated in the text.

Preparation of Hydrolysates

Hydrolysates of aspen, pine and corn stover were prepared by prehydrolysis with 3% SO_2 on fibre as the hydrolysing catalyst at 160°C for 30 min[2], pH raised to 4.8 with lime and saccharified with an enzyme mixture for 48 hours at 50°C. For aspen hydrolysis, cellulase, hemicellulase and cellobiase were used, along with Vitamin B_{12} and trace elements[15,16]. For corn stover the enzyme mixture included also pectinase and glucoamylase[17]. The hydrolysates resulting from this procedure were clarified by centrifugation at 7970 g for 10 min and supernatant used as fermentation substrates.

Extractant Selection

Several extractants were tested for suitability for use in extractive fermentation. Dibutylphthalate was judged the best of these. Freon 113[7] had a very poor distribution coefficient, K_D, defined as the ratio of butanol concentration in the organic layer divided by the butanol concentration in the aqueous layer, being 0.29, whereas K_D for dibutyl phthalate was 1.07. Diethyl phthalate was mildly inhibitory to the growth of the organism, whereas dibutyl phthalate was mildly stimulatory. Three alcohols, 2-ethyl-1-hexanol, 1-octanol and 1-dodecanol, which has better distribution coefficients (5.45, 5.38 and 3.41, respectively) were toxic. Dibutyl phphalate has a boiling point of 340°C, which permits easy recovery of butanol by distillation. However, its low density, 1.56, sets an upper

limit to the sugar concentration which can be used with this extractant.

When distribution coefficients using dibutyl phthalate extractant were measured in a 1:4 ratio of medium: extractant on a solution of butanol; acetone; ethanol in the ratio 6:3:1, which is approximately the ratio frequently found in acetone-butanol fermentation, K_D was found to be for butanol 1.39, acetone 0.63 and ethanol 0.1.

The tests for biocompatability, toxicity and inhibition were performed according to the method of Kelloerup et al.[10].

Extractive Fermentation

Extractive fermentation was carried out in 250 ml shake flasks with Cl. acetobutylicum P262 inoculated in cell concentrations of 6 to 10 g cells/l (lyophilized cell dry weight) unless otherwise indicated, at 35°C in the anaerobic glove chamber on a gently reciprocating shaker. Monot's nutrients were used at twice the concentrations recommended by Monot et al.[13] unless otherwise indicated. Initial pH for these fermentations was 6.5. When selecting extractants, equal volumes of medium and extractant were used, that is 50 ml of each. In some later experiments with dibutylphthalate extractant, where indicated the ratio of medium to extractant was reduced to 1:4, that is 20 ml aqueous medium and 80 ml of dibutylphthalate. The K_D values measured for each solvent under these experimental conditions was as follows: butanol = 1.39, acetone = 0.63 and ethanol = 0.1.

Control Fermentation

These were similar to extractive fermentation, but no extractant was used. Initial cell concentration levels of 6 to 10 g of cells/l were adopted as standard. The effect of various concentrations of Monot's nutrients was studied, and double strength Monot's nutrients was adopted as standard.

Analysis

Solvents in the aqueous phase were analysed by gas chromatography using 3-pentanol as internal standard and using the values of the distribution coefficients. The sum of total concentrations of the solvents in both phases were calculated and are reported. Sugars were analysed by Waters Sugar Analyzer I HPLC using BioRad's Aminex HPX-87P column, water at 65°C as the mobile phase, flow rate 0.5 ml/min, 1-propanol as internal standard and a refractive index detector (Waters 401 RI).

RESULTS

Control (Non-extractive) Fermentations

The course of fermentation of a sugar solution containing 90 g/l glucose (Cl. acetobutylieum P262 4 g/l, double Monot's nutrients) is shown in Figure 1. Fermentation was essentially complete in 24 h, 85% of the sugar being consumed, and solvent production being 22.8 g/l or 0.31 g solvent/g sugar consumed. Of these solvents 60% was butanol, 37% was acetone and 3%

ethanol. At 48 hours, solvent produced were 24.4 g/l, a yield of 0.34 g solvents/g sugar consumed.

Extractive Fermentation

Since high cell concentrations are important for rapid fermentation as shown above, it was felt that these could most easily be achieved by recycling the cells from each fermentation to the next fermentation. Preliminary probes, for which data are not given here, were made in which at the end of each fermentation, cells from the aqueous phase were separated by centrifugation at 7970 g in a Sorvall centrifuge for 10 min and the pellet so obtained served as the sole inoculum into fresh fermentation medium. In a typical series of fermentations, such as for example that shown in Figure 2, which will be further described in the next section, the initial cell concentration was 6.9 g/l, and at 24 h intervals this increased in the presence of dibutylphthalate to 20.0 to 21.6 g cells/l.

Based on the above results, extractive fermentations were carried out in 250 ml shake flasks containing 20 ml of medium and 80 ml of dibutylphthalate, in the anaerobic glove chamber on a gently reciprocating shaker. Initial pH was 6.5.

The results of repeated batch extractive fermentations or glucose are shown in Figure 2. Glucose concentrations were between 83 and 87 g/l at the beginning of each batch, and 95% was consumed within each 24 h period. Solvent yields at 30.8 g/l (concentrations in both aqueous and extractant phase or

total liquid phase) represent 0.37 g solvents/g sugar consumed. The highest single value was 32.1 g/l total solvents. Thus glucose consumption was essentially complete, yields were high and solvent concentrations were over 3 percent, or about one-third higher than in the control experiments.

Figure 3 reports the results of 24 hour repeated batch fermentation with recycle of the bacteria in glucose-xylose substrate in which the ratio of glucose to xylose was 70:30. This ratio was selected as most lignocellulosic materials (especially hard woods) contain about 30% of their total sugar content as pentose sugars, mainly xylose. In these runs, cell concentration increased from 6.5 to 12.5 g/l, and 93% of the total sugars was consumed. Production of solvents was 28 g/l at an efficiency of 0.30 g solvents per g sugar consumed. Thus yields and efficiencies were somewhat lower with the glucose-xylose mixture than experienced with glucose alone.

In Table 1 are shown the results of 24 hour fermentations of aspen, pine and corn stover hydrolysates, in which the cells were recycled to fresh hydrolysate.

Prehydrolysis of aspen wood chips catalysed by SO_2 and followed by enzyme saccharification resulted in the liberation of 64.6 g total sugars/100 g of wood, which is 92% hydrolysis efficiency based on analysis of the wood[16]. The initial total sugar concentration was 73.7 g/l, and of this the average total sugar consumption during 24 h fermentation was 95%. Solvents produced averaged 23 g/l, at an average yield of 0.32 g

solvents/g sugar consumed. This calculates as a yield of 258 l solvents/tonne of aspen wood.

Butanol concentrations were 13.4 g/l, or 58% of the solvents, which as in the run with the mixed glucose-xylose, is rather near the expected 60%, while acetone production was 38%, the excess of acetone over the expected 30% being again at the expense of ethanol production.

Prehydrolysis of pine sawmill residues with SO_2 catalyst followed by enzyme saccharification resulted in the liberation of 71 g total sugars/100 g wood, which is 93% efficiency[15]. In Table 1 are shown the results of 24 hour fermentations of pine wood hydrolysate with the cells recycled to fresh hydrolysate. Total sugar concentration was 135 g/l. This hydrolysate had to be diluted for extractive fermentation. Upon fermentation of the diluted hydrolysate (53 g/l), sugar consumption was 94%, and solvents produced averaged 17.5 g/l, with an average yield of 0.36 g solvents/g sugar consumed. This calculates as 298 l/ solvents/tonne of pine wood.

Prehydrolysis of corn stover with SO_2 catalyst followed by enzyme saccharification resulted in high yields of sugars, 68% on original corn stover, which is 93% of the theoretical maximum based on analysis of the corn stover. Results of repeated 24 h batch fermentations are shown in Table 1. In each 24 h period, 94% of the substrate sugars were utilized. Solvents produced averaged 25.7 g/l, with an efficiency of 0.34 g solvent/g sugar

consumed. This extractive fermentation process resulted in solvent production at the rate of 283 l/tonne of corn stover.

A control experiment with corn stover hydrolysate but no dibutylphthalate as extractant, showed only 74% of substrate utilization in 72 h, suggesting product inhibition, and solvent production was 233 l/tonne corn stover.

DISCUSSION

The advantage of using an extractant during fermentation was clearly demonstrated in the higher concentrations of solvents obtained. In the absence of extractant, the highest values for total solvent concentration obtained in these studies was 24 g/l, from an initial sugar concentration of 86 g/l, while in the presence of extractant 32 g/l total solvents in total liquid volume were obtained from the same initial sugar concentration. In the presence of extractant, the high yield (0.37 g/g sugar consumed) was obtained in 24 h, whereas in its absence the maximum was reached in 48 h.

These results were obtained when high cell concentrations were used, 6 to 10 g cells (dry weight)/l of medium, twice Monot's nutrients, Cl. acetobutylicum P262 as the organism.

Since the extractants with the highest distribution coefficients, namely the three alcohols, are toxic to the microorganism, this would eliminate them from consideration for extractive fermentation, while they may still be useful for recovering products from the beer. All three have boiling

points (184 to 257°C) well above that of butanol (118°C) making recovery of products by fractional distillation possible. Freon 113, which has a low boiling point and therefore offers a low-energy route to recovery of products and extractants[7], has a low distribution coefficient, so low that its usefulness for this purpose must be doubted. The two phthalates studied have distribution coefficients adequate for the purpose of product recovery and are non-toxic. Hence they can be considered for extractive fermentation. The effectiveness of the extractants and their toxicity or non-toxicity are supported by an earlier study on potential extractants for acetone-butanol fermentation done[19]. Our observations suggest that dibutylphthalate stimulated growth, while diethylphthalate was moderately inhibitory to growth. Hence, dibutylphthalate was the extractant of choice.

The advantages of high cell concentrations on rates of acetone-butanol fermentation are demonstrated in these results. There was also considerable cell growth, particularly in the presence of dibutylphthalate, which apparently stimulates cell growth, cell concentrations generally doubling in 24 h of anoxic fermentation. This work also demonstrated that the cells at the end of fermentation could be recovered by centrifugation and re-used - "recycled" - to the succeeding fermentation. This continued viability and fermentative activity suggest that prolonged continuous operation should be readily achievable.

All extractive fermentations of pure sugars, glucose and glucose-xylose mixtures at concentrations between 80 to 100 g/l, were completed in 24 h with solvent yields of 28 to 30 g/l, compared with 18 to 20 g/l for controls in which the extractant was not present, an increase of about 50% in sugar utilization. The yields were slightly better with glucose than with glucose-xylose mixtures, suggesting poorer conversion of the xylose by this organism[3].

The hydrolysates of the lignocellulosics, which are a mixture of hexose and pentose sugars, fermented very well, with about 95% of total sugars converted to solvents with conversion factors about 85% of theory. Yields of solvent from aspen wood, pine wood and from corn stover were 258, 298 and 283 l/tonne of feedstock (on a dry basis), respectively. Such high yields of products from inexpensive agricultural and forest industries waste materials offers the prospect of economic production of fermentation acetone-butanol for use as co-solvent in methanol motor fuel.

ACKNOWLEDGEMENTS

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lignocellulosic hydrolysates were supplied by Dr. Sarad Parekh and Catherine Seagrave of this laboratory.

REFERENCES

1) Weizman, C. British Patent 4845 (1919, U.S. Patent 1,315,584), (1935).

2) Wayman, M., Tallevi, A., Winsborrow, B.: Biomass., 6, 183 (1984a).

3) Wayman, M., Yu, S.: Biotechnol. Lett., 7, 255 (1985).

4) Essien, D., Pyle, D.L.: Process Biochemistry., Aug. 18, 31 (1983).

5) Minier, M., Goma, G.: Biotechnol. Lett., 3, 405 (1981).

6) Minier, M., Goma, G.: Biotechnol. Bioeng., 24, 1565 (1982).

7) Wayman, M., Husted, G.R., Santangelo, J.D., Levy, S.: Proceedings VI International Symposium on Alcohol Fuels Technology, 2, 239 (1984).

8) Taya, M., Ishii, S., Kobayashi, T.: J. Ferment. Technol., 66(2), 18 (1985).

9) Ishii, S., Taya, M., Kobayashi, T.: J. Chem. Eng. Jap., 18(2), 125 (1985).

10) Pye, E.K. and Humphrey, A.E.: Univ. Penn., Annual Report to U.S. Dept. of Energy, (Sept. 1978-Sept. 1979).

11) Kormendy, A., Wayman, M.: Can. J. Microbiol., 20, 225 (1974).

12) Long, S., Jones, D.T. Woods, D.R.: Appl. Environ. Microbiol., 45, 1389 (1983).

13) Monot, F., Martin, J.R., Petitdemange, H., Gay, R.: Appl. Environ. Microbiol., 44, 1318 (1982).

14) Yu, S.: Ph.D. Thesis, University of Toronto, (1986).

15) Wayman, M., Parekh, S., Chornet, E., Overend, R.: Biotechnol. Lett., 8, 749 (1986).

16) Parekh, S.R., Yu, S., Wayman, M.: Eur. J. Appl. Microbiol. Biotechnol. (In press).

17) Wayman, M., Parekh, S.: Biotechnol. Lett., 7, 909 (1985).

18) Kollerup, F., Daugulis, A.J.: Can. J. Chem. Eng., 63, 919 (1985).

19) Moreira, A.R.: Organic Chemicals from Biomass (ed. D.L. Wise), p. 385. Benjamin/Cummings, Melo Park, California (1983).

FIGURES

FIGURE 1: Acetone-butanol fermentation by Cl. acetobutylicum P262 using initial inoculum 4.0 g/l.
(Δ) glucose, (o) total solvents

FIGURE 2: Extractive acetone-butanol fermentation of glucose by Cl. acetobutylicum P262 using dibutylphthalate as extractant.
Control, (Δ) glucose, (o) total solvents
Extractive fermentation (▲) glucose, (●) total solvents

FIGURE 3: Extractive acetone-butanol fermentation of glucose-xylose (70:30) mixture by Cl. acetobutylicum P262 using dibutylphthalate as extractant.
Control, (Δ) total reducing sugars, (o) total solvents
Extractive fermentation (▲) total reducing sugars, (●) total solvents

TABLE 1

24 h repeated batch extractive acetone-butanol fermentation of hydrolysates of aspen wood, pine sawmill residue and corn stover by Cl. acetobutylicum P262

	Total reducing sugars g/l	Sugar consumption %	Total Solvents g/l	Butanol g/l	Yield g solvents/ g sugar consumed	Total solvents l/tonne	% of theory on wood
Aspen							
1	78	97	24.6	13.4	.32	255	77
2	69	93	20.1	11.7	.31		
3	75	95	24.2	15.1	.34		
Control[1]	78	83	20.8	11.3	.32	216	65
Pine							
1	53	93	17.63	10.52	.36	298	83.5
2	53	93	17.93	11.16	.36		
Control[1]	53	77	13.97	9.09	.34	231	66
Corn Stover							
1	81	94	25.6	15.0	.34	283	84
2	81	93	25.8	15.2	.34		
3	83	94	25.7	15.1	.34		
Control[1]	81	74	21.9	12.3	.36	233	69

[1] Control expt. was non-extrative fermentation.

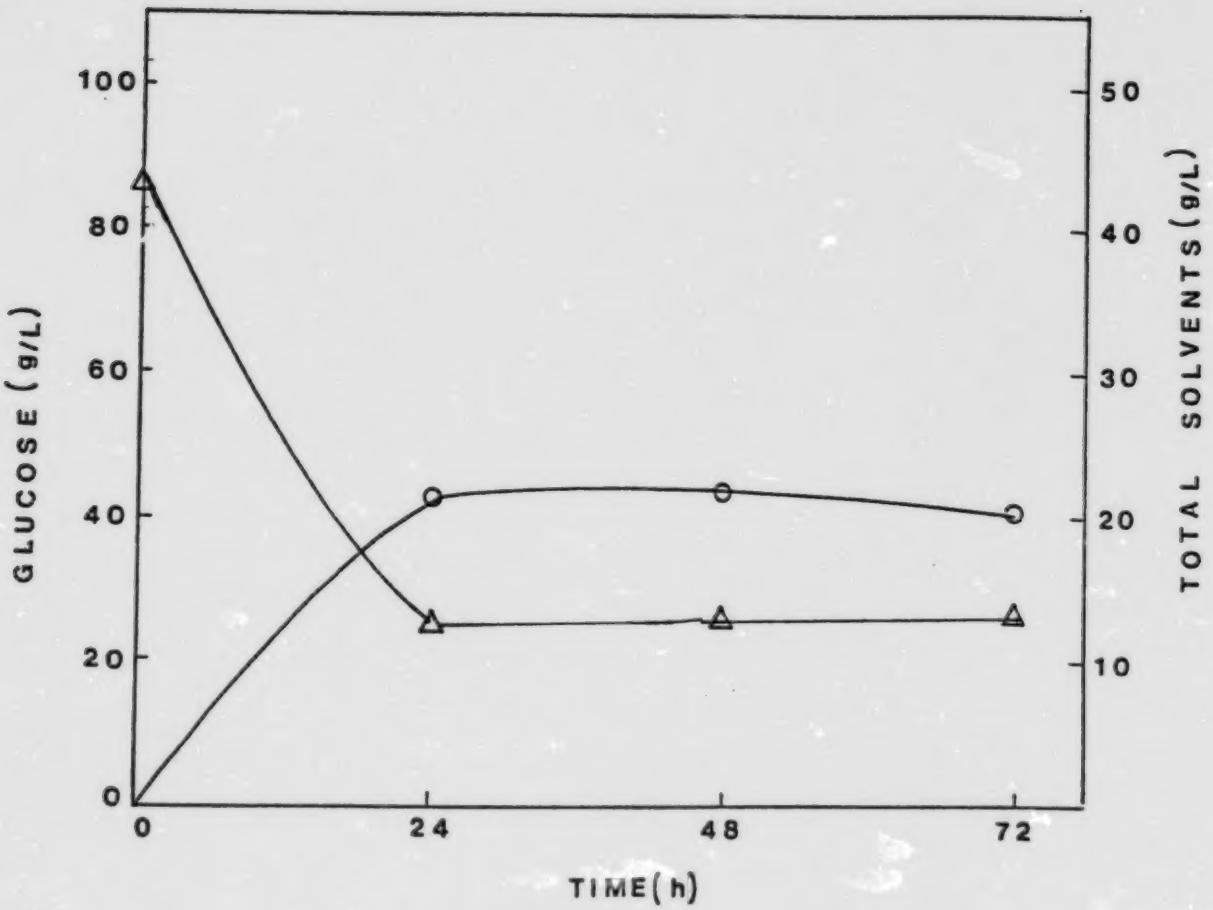
GLUCOSE (g/L)
TOTAL SOLVENTS (g/L)
TIME (h)
0
20
40
60
80
100
10
20
30
40
50
0
24
48
72

Fig 2

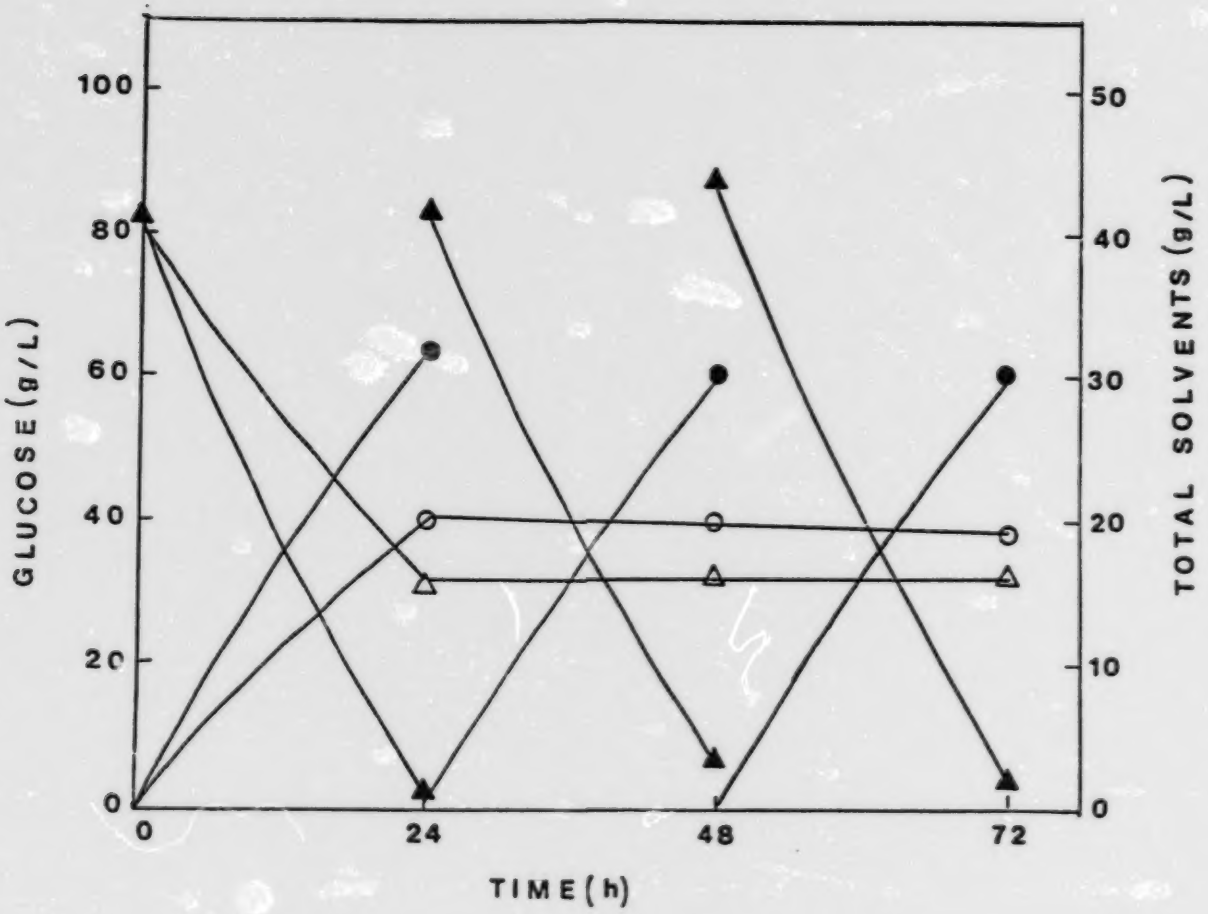
GLUCOSE (g/L)
TOTAL SOLVENTS (g/L)
TIME (h)
0
20
40
60
80
100
0
10
20
30
40
50
0
24
48
72

Fig. 3

TOTAL REDUCING SUGARS (g/L)
TOTAL SOLVENTS (g/L)
TIME (h)
0
20
40
60
80
100
10
30
50
24
48
72

10.11

Fermentation of Xylose to Ethanol by *Candida shehatae*

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Summary

Ethanol fermentation of xylose by *Candida shehatae* in a semiaerobic mode at pH 5.0 and 30°C with 2 g/L inoculum produced 0.30–0.39 g ethanol/g xylose consumed, with xylose concentrations of 30–200 g/L. Peak ethanol concentrations were obtained in 24 h at 30 g/L but required 72 h at 120 g/L. Oxygen concentration cycled between 8.4 and 0.7 ppm. Addition of estimated ethanol consumption by the yeast to measured ethanol production resulted in a calculated production rate of 0.38–0.45 g ethanol/g xylose consumed. Anaerobic fermentation was slower, being 73% complete in 70 h. Results with seven other promising yeasts are reported. Keywords: xylose fermentation to ethanol, *Candida shehatae*, semiaerobic fermentation, lingnocellulosics.

INTRODUCTION

Agroindustrial and agricultural crop residues and hardwoods have a high content of xylan, and the economy of ethanol production from such biomass raw materials, lignocellulosics, would be much improved by successful fermentation of the xylose in their hydrolysates. To date, such fermentation has been found to be slow, ethanol concentrations low, and ethanol yields poor. This article reports the fermentation of D-xylose to ethanol by a strain of *Candida shehatae*. Previous studies of *C. shehatae*[1,2] have shown that this species is a more efficient xylose fermenter than the more-researched *Pachysolen tannophilus*[3-9] Further, Toivola et al[10] concluded that *C. shehatae* is one of the few potential candidates for industrial fermentation of wood sugars. Since aeration during fermentaton has been reported to enhance ethanol production,[1-3,11] fermentations were run under semiaerobic and anaerobic conditions. In some of these runs oxygen concentration was monitored during fermentation. Also, since air is considered to be a hydrogen acceptor in this fermentation, the effect of an organic hydrogen acceptor, anthraquinone, was investigated. Finally, fermentations of xylose were carried out using seven promising yeast strains.[10]

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MATERIALS AND METHODS

Microorganisms

The strain of *Candida shehatae* used was ATCC 22984, supplied by Dr. Thomas W. Jeffries, USDA Forest Products Laboratory, Madison, Wisconsin.[12] *C. shehatae* CBS 5813, CBS 4705, *C. tennuis* CBS 4113, CBS 4285, CBS 4435, *Pichia segobiensis* CBS 6857, and *P. stipitis* CBS 5773 were obtained from the Centraalbureau voor Schimmelcultures (CBS), Yeast Division, Delft, The Netherlands.

Media and Culture Conditions

All strains were maintained on yeast extract–malt (YM) agar slants with xylose as the carbon source. The cultivation medium consisted of the following, per liter: D-xylose, BDH analytical grade, 20 g; peptone, 5 g; yeast extract, 3 g; and malt extract, 3 g. After adjusting the pH to 5.5, solutions of xylose and other components were autoclaved and combined after cooling, at which time the pH was 5.0. The fermentation medium was similar, except that ammonium sulfate, 5 g, was substituted for peptone, and the xylose concentration was varied from 30 to 200/L.

Inocula were prepared by yeast cultivation in 250-mL shake flasks in 100 mL media as intended for the fermentation runs. After 3 days cells were harvested by centrifugation at 10,400*g* for 8 min and washed once with 0.05*M* NaCl. The pellet was resuspended and used to inoculate a fermentation flask. Initial cell concentration was 1–2 g/L.

Modified 250-mL side arm flasks were used for batch fermentations. The side arms were cut off at 1.5 cm and the openings closed with suba seals. This facilitated sampling by syringe. Under semiaerobic conditions a porous foam closure was used. Under anaerobic conditions a fermentation stopper with water seal was used. Fermentations were conducted in a New Brunswick G27 controlled environment incubator rotary shaker operating at 150 rpm and 30°C.

Analysis

Xylose was determined by the dinitrosalicylic acid (DNS) method of Miller.[13] Ethanol was determined from cell-free samples using a Hewlett Packard 5700 gas chromatograph equipped with a 2-m Poropak Q column. The operating temperature was 150°C, and *n*-propanol was used as an internal standard. The pH, which normally fell from 5.0 at the start of fermentation to below 3.0 at the point of maximum ethanol concentration, and the cell concentration (OD at 660 nm) were monitored at suitable intervals. Dissolved oxygen was measured using a Chemtrix Type 30 oxygen meter.

RESULTS

Candida shehatae ATCC 22984

Semiaerobic fermentations were conducted using various initial xylose concentrations ranging from 31.2 to 206.8 g/L when using *C. shehatae*. Xylose consumption and ethanol concentration are shown in Figure 1 and are

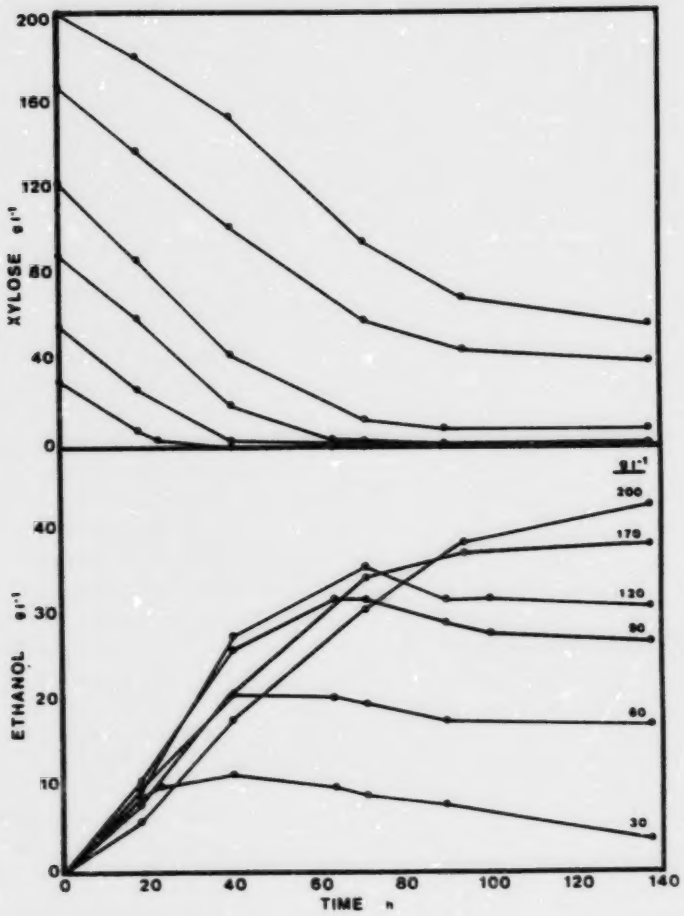

Fig. 1. Semiaerobic fermentation of D-xylose by *C. shehatae*, initial cell concentration 2 g L initial pH 5.0, 30°C, 100 mL medium in 250-mL shake flasks, 150 rpm

summarized in Table I. In all cases xylose consumption was rapid and essentially complete in 23 h at an initial concentration of 31.2 g/L, in 40 h at 57.2 g/L, in 64 h at 90.5 g/L, and in 71 h at 124.0 g/L. At initial xylose concentrations of 170.3 and 206.8 g/L, xylose consumption slowed markedly at 94 h, when about 75% of the xylose had been consumed. Ethanol production was rapid at first, and

TABLE I

Semiaerobic Fermentation of D-xylose by *Candida shehatae*.[a]

Initial substrate concentration (g/L)	Time (h)	Ethanol yield $\left(\frac{\text{g ethanol}}{\text{g xylose consumed}}\right)$
31.2	23	0.37
57.2	40	0.39
90.5	64	0.37
124.0	71	0.32
170.3	94	0.30
206.8	94	0.28

[a] Initial cell concentration 2 g/L, initial pH 5.0, 30°C, 100 mL medium in 250-mL shake flasks, 150 rpm.

the concentration reached a maximum in each case of up to 120 g/L and declined thereafter. The time at which the ethanol concentration reached a maximum was the same as the time at which xylose consumption was greatest, that is, at the times mentioned just above for each initial xylose concentration. The decrease in ethanol concentration can be attributed to ethanol consumption by the yeast under semiaerobic conditions and to evaporation. Of these two, the former is more significant.

Ethanol concentration reached a maximum at 140 h when the initial xylose concentration was 170.3 g/L and at 180 h or more when the initial xylose concentration was 206.8 g/L. These maxima may result from product inhibition, although ethanol consumption may be equally important. The level of ethanol concentration at which product inhibition becomes important for *C. shehatae* has yet to be determined.

The yields of ethanol at the peak of each curve are shown in Table I and are 0.37–0.39 g ethanol/g xylose consumed for initial xylose concentrations up to 90.5 g/L and at 0.32 or less at higher initial concentrations. The theoretical yield of ethanol from xylose is 0.465 g/g.[14] Yields at the lower concentrations represent 80–84% of the theoretical. These less than theoretical yields may be attributed largely to consumption of ethanol by the yeast under these semiaerobic conditions. An estimate of ethanol consumption may be made from the slope of the ethanol concentration curve for the initial xylose concentration of 31.2 g/L after 40 h. This amounts to 0.08 g/Lh. If ethanol concentrations are corrected for ethanol consumption, the calculated results are shown in Table II.

It is seen from Table II that the yields have improved significantly to the range

of 0.38–0.45 g ethanol/g xylose consumed, the best result being 97% of theory. No measurements of xylitol, glycerol, or other possible by-products were made to account for the deficiency.

TABLE II

Semiaerobic Fermentation of D-xylose by *Candida shehatae*.[a]

Initial xylose concentration (g/L)	Time (h)	Ethanol concentration measured (g/L)	Ethanol concentration corrected for ethanol consumption (g/L)	Corrected yield $\left(\frac{\text{g ethanol}}{\text{g xylose consumed}}\right)$
31.2	23	10.3	12.1	0.43
57.2	40	21.0	24.2	0.45
90.5	64	32.2	37.3	0.43
124.0	71	35.9	41.6	0.38

[a] Initial pH 5.0, 30°C, 100 mL medium in 250-mL shake flasks, 150 rpm.

Anaerobic fermentations were also run with *C. shehatae* with 80–100 g/L xylose. The results are shown in Figure 2. The rate of fermentation was much slower even though copious gas evolution was observed. After 70 h only 73% of the available xylose was consumed. This resulted in a yield of 0.31 g ethanol/g xylose consumed. In the control semiaerobic fermentation at 90 g/L consumption of xylose was essentially complete at 64 h, and the yield was 0.37 g ethanol/g xylose consumed.

Bruinenberg et al.[13] have reported that the presence of a hydrogen acceptor enhanced anaerobic fermentation by *C. utilis*. A hydrogen acceptor, anthraquinone, has proven to be a remarkably effective catalyst in wood pulping. When anthraquinone was added to xylose fermentation at 10 g/L, no improvement in the rate of fermentation was seen. Anthraquinone also had no observable effect on semiaerobic fermentation. It was thought that the ethanol concentration might be maintained if fermentation conditions were changed from semiaerobic to anaerobic after most of the ethanol was formed. This change was made at 48 h, and there is some evidence that loss of ethanol ceased at that point.

The concentration of dissolved oxygen during semiaerobic fermentation of 100 g/L xylose by *C. shehatae* is shown in Figure 3. Oxygen concentration was initially 8.4 ppm, being 100% saturation, and this fell to 2.0 ppm during the first 20 h. At 44 h, when the ethanol concentration was at its maximum, oxygen concentration was 0.7 ppm, about 8% of saturation. Thereafter, as fermentation slowed and ceased, oxygen concentration increased. Cell concentration increased steadily during this semiaerobic fermentation, which represents diversion of xylose assimilation from ethanol formation to yeast growth. Some of this growth may be attributed to alcohol consumption[14,15] under the semiaerobic conditions employed.

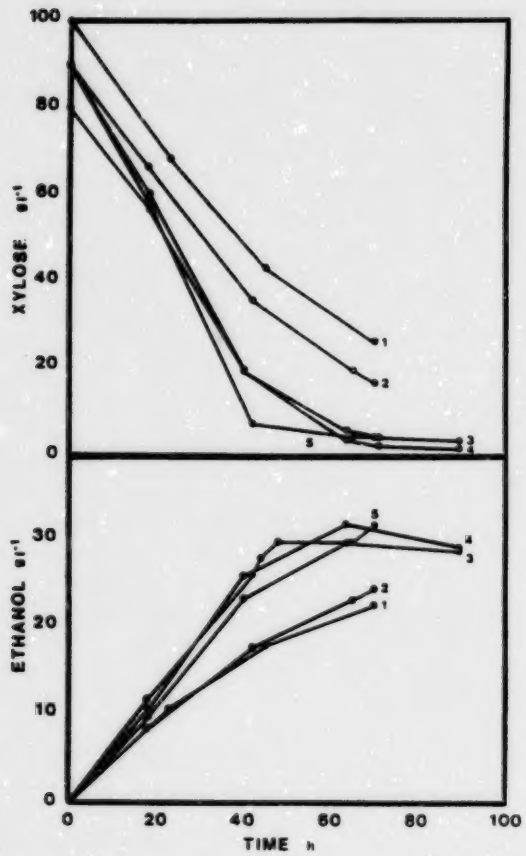

Fig. 2. Fermentation of D-xylose by *C. shehatae*, conditions as in Figure 1. (1) Anaerobic fermentation, (2) anaerobic fermentation with the addition of anthraquinone, (3) semiaerobic fermentation with the addition of anthraquinone, (4) semiaerobic fermentation (control), and (5) semiaerobic fermentation changed to anaerobic fermentation after 48 hr.

Other Yeasts

In the paper by Toivola et al.[10] 200 glucose-fermenting, xylose-assimilating yeast species were tested for ethanolic fermentation of xylose. Of these, we selected seven strains that could be considered the most promising, including two strains of *C. shehatae*, three of *C. tennuis*, and one each of *Pichia segobiensis* and *P. stipitis*. These were used to ferment 120 g/L D-xylose semiaerobically, with the results shown in Figure 4. This figure also contains a control fermentation with *C. shehatae* ATCC 22984. It is apparent from Figure 4 and the results summarized in Table III, that *C. shehatae* CBS 5813 is almost as good as

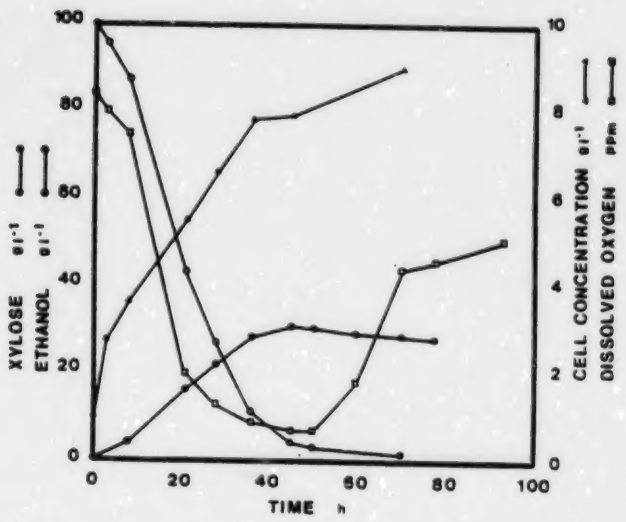

Fig. 3. Semiaerobic fermentation of 100 g/L D-xylose by *C. shehatae*, conditions as in Figure 1, monitoring dissolved oxygen.

TABLE III

Semiaerobic Fermentation of 120 g/L D-xylose.[a]

Species	Strain	Substrate consumed (%)	Ethanol yield $\left(\frac{\text{g ethanol}}{\text{g xylose consumed}}\right)$
C. shehatae	CBS 5813	90	0.30
	CBS 4705	55	0.39
C. tenuis	CBS 4113	45	0.14
	CBS 4285	45	0.16
	CBS 4435	70	0.21
P. segobiensis	CBS 6857	40	0.16
P. stipitis	CBS 5773	40	0.36

[a] Initial cell concentration 2 g/L, initial pH 5.0, 30°C, 100 mL medium in 250-mL shake flasks, 150 rpm after 72 hours. The results for CBS 5813 were taken at 67.5 h.

the ATCC strain in rate of fermentation and ethanol production, while the others are less effective under our conditions.

DISCUSSION

The results of this study confirm the industrial potential of *C. shehatae* as a fermenter of xylose to ethanol. Semiaerobic fermentations were rapid, producing

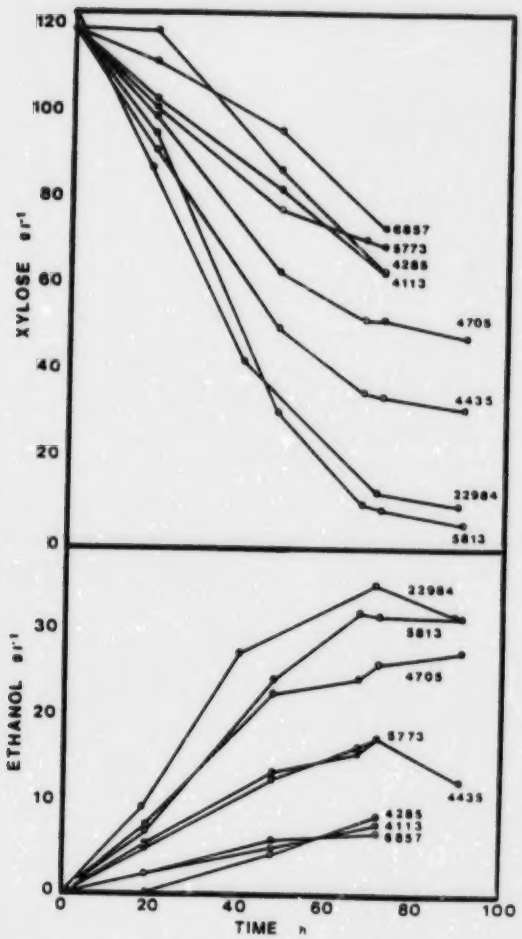

Fig. 4. Semiaerobic fermentation of 120 g/L D-xylose by seven promising yeast strains, conditions as in Figure 1.

ethanol yields up to 84% of the theoretical maximum. Initial xylose concentrations of up to 170 g/L produced no substrate inhibition as evidenced by the rapid initial fermentation rate, although there may be a question of product inhibition at concentrations of ethanol above 40 g/L. This microorganism is able to ferment xylose anaerobically but produces ethanol at only two-thirds the rate obtained using semiaerobic conditions.

Semiaerobic fermentation can be considered to take place in three phases as illustrated in Figure 3. During the first phase cell growth and ethanol production consume xylose rapidly, and the dissolved oxygen content decreases. This

oxygen decrease may be attributed to the consumption of oxygen during cell growth and to the production of CO_2 as a product of fermentation. The latter creates a positive pressure in the fermentation vessel. This positive pressure minimizes two-way gas transport through the porous foam plug. The second phase occurs when the dissolved oxygen falls below 1 ppm. This concentration is insufficient to sustain cell growth. At this time the fermentation rate decreases and ethanol concentration reaches a maximum. When fermentation and CO_2 production virtually cease, air once again diffuses into the fermentation flask. During phase three oxygenation of the fermentation broth results in renewed cellular activity and cell growth. With xylose nearly depleted, ethanol is readily assimilated.[16] Du Preez and van der Walt[1] have shown that after xylose is consumed, the rate of ethanol assimilation increases with the rate of aeration. Although some loss of ethanol occurs by evaporation, ethanol losses can be largely attributed to assimilation. This hypothesis is supported by the observed increase in cell concentration.

Although Suihko and Drazic[17] are doubtful about the industrial feasibility of aerobic or semiaerobic fermentation, there are good examples of aerated fermenters in industrial production of yeast on quite a large scale.[18] The only additional factor needed for semiaerobic fermentation is control of dissolved oxygen through a predetermined cycle for batch fermentation and perhaps a cascade mode of operation with appropriate dissolved oxygen levels in each stage. These are not serious difficulties from an engineering point of view.

Bruinenburg et al.[15] ascribed the inability of most facultatively fermentative xylose-assimilating yeasts to ferment xylose anaerobically to a metabolic block in the xylose metabolic pathway. The conversion of xylose to xylulose via xylitol results in a surplus of NADH. This effectively blocks off the formation of xylulose and leads to the accumulation xylitol at the expense of ethanol formation. The addition of acetoin, a hydrogen acceptor that can oxidize NADH, enabled *C. utilis* to ferment xylose anaerobically.[14] Recently Toivola et al.[10] reported that a few species of yeast were able to ferment xylose anaerobically. Bruinenberg et al.[15] correlated this ability with the presence of a NADH-linked xylose reductase. In our work anaerobic fermentation of xylose to ethanol by *C. shehatae* took place, albeit at a slower rate than semiaerobically, as shown in Figure 2, suggesting that the necessary enzymes, including the NADH-linked xylose reductase of Bruinenburg, are present or are induced in this yeast. The slower rate of fermentation suggests that the formation of this enzyme in *C. shehatae* may be induced by the presence of xylose under anaerobic conditions. Under semiaerobic conditions, dissolved oxygen in the fermentation medium decreased rapidly in the early hours of fermentation, as shown in Figure 3, resulting in a near-anaerobic atmosphere.

The addition of anthraquinone, a hydrogen acceptor, in place of oxygen did not significantly increase the fermentative ability of *C. shehatae*. This may have been a poor choice of hydrogen acceptor, and better results may have been obtained with acetoin[15] or the respiratory inhibitor azide.[19]

This work suggests a possible explanation for a difference between *C.*

shehatae and other xylose-assimilating yeasts in that the necessary enzymes for anaerobic fermentation of xylose to ethanol are inducible rather easily in this yeast by xylose under anaerobic conditions; for example, the NADH-linked xylose reductase could be induced more rapidly or in greater quantity than in other xylose-fermenting yeasts.

CONCLUSIONS

1. *Candida shehatae* ATCC 22984 has the potential of being adopted for the industrial fermentation of pentoses to ethanol. In this respect, it is superior to other pentose-fermenting yeast strains studied here.
2. The semiaerobic mode is necessary for rapid fermentation rates and good ethanol yields. In this mode oxygen-saturated medium becomes depleted of oxygen during fermentation. When fermentation ceases due to exhaustion of the sugar substrate, assimilation of ethanol and yeast growth reduce ethanol concentrations significantly.
3. This work suggests that the enzymes necessary for anaerobic fermentation of xylose to ethanol by *C. shehatae* are inducible rather easily compared to other yeasts.
4. This study leaves unanswered a number of questions: What is the best dissolved oxygen control program for optimal ethanol production and conservation? Can the fermentation take place in continuous equipment, and if so, what arrangements are required for dissolved oxygen control, for example, cascade or tubular fermenters? Is the rate of fermentation now limited by the level of inoculum, and if so, would this be increased by higher inoculum levels, tower fermentation with sedimented yeast or immobilized yeast? At what concentration does ethanol become inhibitory to this yeast? Can conditions be identified for rapid induction of the necessary enzymes to enable anaerobic fermentation to take place at acceptable rates and yields? These questions will be studied as this research progresses.

We are grateful to Dr. Thomas W. Jeffries, USDA Forest Products Laboratory, Madison, Wisconsin, for drawing our attention to the potential of *C. shehatae* and providing a culture for our use. We also acknowledge funding from the Natural Sciences and Engineering Research Council of Canada, and Energy, Mines and Resources Canada.

References

[1] J. C. du Preez and J. P. van der Walt, *Biotechnol. Lett.*, **5**, 357 (1983).
[2] J. C. du Preez, B. A. Prior, and M. T. Monteiro, *Appl. Microbiol. Biotechnol.*, **19**, 261 (1984).
[3] H. Schneider, P. Y. Wang, Y. K. Chan, and R. Maleazka, *Biotechnol. Lett.*, **3**, 89 (1981).
[4] P. J. Slininger, R. J. Bothast, J. E. Van Cauwenberge, and C. P. Kurtzman, *Biotechnol. Bioeng.*, **24**, 371 (1982).
[5] D. Debus, H. Methner, D. Schulze, and H. Dellweg, *Eur. J. Appl. Microbiol. Biotechnol.*, **17**, 287 (1983).

[6] K. F. Deverell, *Biotechnol. Lett.*, **7**, 475 (1983).
[7] B. Mutze and C. Wandrey, *Biotechnol. Lett.*, **5**, 633 (1983).
[8] T. W. Jeffries, *BioTechnology*, **8**, 503 (1983).
[9] H. Dellweg, M. Rizzi, H. Methner, and D. Debus, *Biotechnol. Lett.*, **6**, 395 (1984).
[10] A. Toivola, D. Yarrow, E. van den Bosch, J. P. van Dijken, and W. A. Scheffers, *Appl. Environ. Microbiol.*, **6**, 1221 (1984).
[11] T. W. Jeffries, *Biotechnol. Lett.*, **3**, 213 (1981).
[12] T. W. Jeffries, *Biotechnol. Lett.*, **6**, 777 (1984).
[13] G. L. Miller, *Anal. Chem.*, **3**, 426 (1959).
[14] C-S. Gong and G. T. Tsao, *Proceedings International Symposium on Ethanol from Biomass*, H. E. Duckworth and E. A. Thompson, Ed. (Royal Society of Canada, Ottawa, (1983), pp. 525–555.
[15] P. M. Bruinenberg, P. H. M. de Bot, J. P. van Dijken, and W. A. Scheffers, *Eur. J. Appl. Microbiol. Biotechnol.*, **18**, 287 (1983); **19**, 256 (1984).
[16] M. Wayman, *Can. J. Microbiol.*, **20**, 1675 (1974).
[17] M. L. Suihko and M. Drazic, *Biotechnol. Lett.*, **5**, 107 (1983).
[18] W. Sittig, *Chemtech*, **13** (10), 606 (1983).
[19] E. Lohmeier-Vogel, Birgitta Jönsson, and Barbel Hahn-Hagerdal, Annals of the New York Academy of Sciences, **434**, 152 (1984).

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REFERENCES

1. JOHANSSON, B., MJÖBERG, J., SANDSTRÖM, P. and TEDER, A. *Svensk Papperstidn.* 87 (10):30 (1984.)
2. SJÖBLOM, K., HARTLER, N., MJÖBERG, J. and SJÖDIN, L. *Tappi* 66 (9):97 (1983).
3. TEDER, A. and OLM, L. *Paperi ja Puu* 63 (4a):315 (1981).
4. BACKLUND, E.Å. TAPPI Pulping Conf., San Francisco, USA, November 1984, reprints p. 1.
5. NORDÉN, S. and TEDER, A. *Tappi* 62 (7):49 (1979).
6. JACK, W.Q. and FELLER, L.D. *Pulp & Paper Can.* 68 (9):T461 (1967).
7. ANNERGREN, G. et al. US Pat. 856664 (Dec 2, 1977).
8. RAPSON, W.H., ANDERSSON, C.B. and REEVE, D. *Pulp & Paper Can.* 78 (6):T137 (1977).
9. GERMGÅRD, U. *Tappi* 65 (11):81 (1982).
10. GERMGÅRD, U. and KARLSSON, R-M. *Paperi ja Puu* (11):627 (1984).
11. AXEGÅRD, P. TAPPI Pulping Conf., San Francisco, USA, November 1984, reprints p. 353.
12. GERMGÅRD, U. and KARLSSON, R-M. TAPPI Pulping Conf., San Francisco, USA, November 1984, reprints p. 183.
13. DU MANOIR, J., *Pulp & Paper Can.* 83 (2) T58 (1982).
14. ANNERGREN, G. and LINDBLAD, P.O. Paper presented at the Ekman Days in Stockholm, Sweden, January 1974.
15. GERMGÅRD, U., TEDER, A. and TORMUND, D. *Paperi ja Puu* (4):264 (1983).
16. GERMGÅRD, U. and KARLSSON, R-M. *Svensk Papperstidn.* 89 (15):R133 (1986).
17. Environmentally harmonized production of bleached pulp. Final report from SSVL, Stockholm, (1982). IPK, Box 8309, S-104 20 Stockholm.
18. GERMGÅRD, U., KRINGSTAD, K. et al. Paper presented at the TAPPI Oxygen Delignification Conf in San Francisco, USA, November 1984.
19. RENBERG, L. et al. *Chemosphere* 9:143 (1980).
20. NEILSSON, A.H. et al, *Appl. Environ. Microbiol.* 45:774 (1983).
21. VOSS, R.H., WEARING, J.T., WONG, A. et al. *Pulp & Paper Can.* 81 (12):T367 (1980).
22. KUTNEY, G.W., HOLTON, H.H., ANDREWS, D.H., du MANOIR, J.R. and DONNINI, G.P. *Pulp & Paper Can.* 85(5):T95 (1984).
23. SJÖSTRÖM, L.R., RÅDESTRÖM, R. and LINDSTRÖM, K. *Svensk Papperstidn.* 85 (3):R7 (1982).
24. LINDSTRÖM, K. Swedish Forest Products Research Laboratory, Box 5604, S-114 86 Stockholm, Sweden. To be published in *Env. Sci. & Techn.*
25. VOSS, R.H. et al. CPAR Project Report No. 828, Canada, June 1979.

Fermentation of Spent Sulphite Liquor to Butanol and Ethanol

S. YU and M. WAYMAN

With the objective of achieving complete desugaring ahead of vanillin production, spent sulphite liquor (s.s.l.) has been fermented to butanol by two strains of Clostridium *and to ethanol by* Candida shehatae, *these micro-organisms being chosen for their ability to ferment both hexoses and pentoses. For butanol fermentation, s.s.l. was first steam stripped to pH 4.5 or higher in order to remove more than 90% of volatile inhibitors present in the liquor.* Cl. butyricum *ATCC 8260 and* Cl. acetobutylicum *NRRL 594 were successfully adapted to s.s.l. by a series of subculturing transfers. While sugar consumptions (73-84%) and solvent production (0.18-0.25 g/g of initial sugar) were lower than that experienced with pure sugars (96% and 0.35 g/g of sugar, respectively), this fermentation was more complete than industrial ethanol fermentation by* Saccharomyces cerevisiae. *For ethanol fermentation* C. shehatae *ATCC 22984 was able to ferment s.s.l. steam stripped from pH 2 to pH 3 to 3.4 without adaptation. By recycling the yeast in s.s.l. many times, and by the use of high cell concentration, 13-19 g/L, fermentation was complete in 12 to 18 h, 95.5% of the total sugars present were consumed, and efficiency was 0.46 g ethanol/g initial sugar concentration, compared to 65.4% sugar removal and 0.35 g ethanol/g initial sugar concentration with* S. cerevisiae. *Thus 33% more alcohol was produced by this yeast, and 46% more sugar was removed from the s.s.l.*

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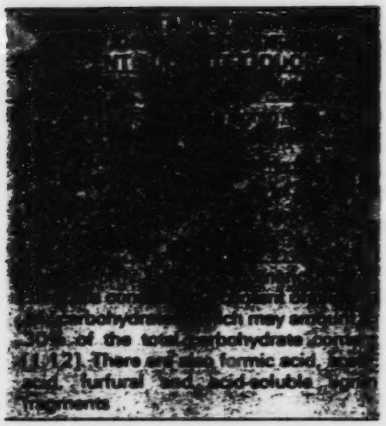
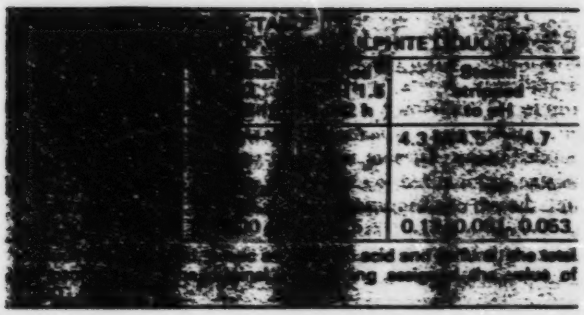
INTRODUCTION

While the importance of the sulphite process for the pulping of wood has been declining in recent years relative to other processes, it is still important commercially both for papermaking and for chemical byproducts [1]. The Ontario Paper Company mill at Thorold, Ontario, manufactures sulphite pulp at the rate of about 160 tons per day as the chemical pulp component of its large newsprint production. At this mill, the spent sulphite liquor, which is the solution of wood solubles generated during the chemical pulping process, is separated from the pulp which is largely cellulose, and processed to make several other products. Since more than half of the wood is dissolved during sulphite pulping, it is clear that somewhat more than 160 tons





of non-cellulosic chemicals in addition to the pulping chemicals are contained in the spent sulphite liquor produced daily.

In addition to inorganic chemicals such as SO_2, the liquor contains lignosulphonic acids, and wood sugars. Analysis of a recent sample revealed the organic composition shown in Table I.

Following a partial removal of free SO_2 by heating, the liquor is cooled, brought to pH 4.5 with lime, a small amount of diammonium phosphate and urea are added, and then yeast, and the liquor is fermented to ethanol. The yeast used is *Saccharomyces cerevisiae,* ordinary commercial bakers' yeast, entirely recycled from previous batch fermentations except when the system has been shut down for vacations or maintenance, in which case freshly bought yeast is added. Fermentation takes 12 to 18 h, and the resulting ethanol is recovered, purified by successive distillations and sold as industrial alcohol.

The residue from the distillation, the alcohol plant effluent, contains lignin and unfermented sugar. This is then processed further, by adding sodium hydroxide and a catalyst and heating under pressure with air to oxidize the lignin to vanillin. Annual production in this mill is about 6 million pounds of vanillin per year which may be the largest output of any such facility in the world.

The fermentation of spent sulphite liquor to ethanol is a long-established process, having been practiced in Scandinavia and continental Europe for many years.

When hardwoods are pulped, as in most continental European pulp mills, the composition of the spent sulphite liquor is quite different, having a much higher content of pentose sugars relative to hexose sugars. Such pentose-rich spent sulphite liquors have been considered more suitable substrates for growing yeast than fermenting to alcohol, for example at the Waldhof sulphite mill, where the widely used Waldhof fermenter was first developed and where the yeast formed the basis for a pharmaceutical chemical industry; or at Attisholz, in Switzerland, where fermentation of the hexoses to alcohol leaves still bottoms rich in pentoses, which are substrates for growth of yeasts which are sold primarily as cattle feed. While *Candida utilis* has traditionally been the species of choice for such purposes, in modern practice mixed cultures are also used. The option of utilizing spent sulphite liquor sugars for yeast growth is open only to a small number of pulp mills world wide because of limited markets for "single cell protein" cattle feed.

The composition of the spent sulphur liquor from Ontario Paper Company shown in Table I is higher in mannose than in xylose, and is in fact about 72% hexoses and 28% pentoses, reflecting the fact that the mill pulps mainly coniferous woods such as spruce, balsam and pine. Had hardwoods such as poplar or birch been pulped, the spent sulphite liquor would have contained about 50% of each of hexoses and pentoses, resulting in lower alcohol yields. In addition, the lignin from coniferous woods yields a purer vanillin than that from hardwoods. For these reasons coniferous woods are preferred in such operations. Since the present fermentation to ethanol utilizes only the hexoses, the pentose sugars remain, and are present in the vanillin reactor. Here they are oxidized to organic acids which consume sodium hydroxide and generally add to the cost of vanillin production with no identifiable benefit. For this reason a fermentation process for utilization of both hexoses and pentoses is highly desirable.

We have approached this problem from two different directions. In one approach, we have attempted fermentation of the spent sulphite liquor to butanol by *Clostridium acetobutylicum* and by *Cl. butyricum* strains which utilize both hexoses and pentoses. In a previous paper [2], we reported the fermentation of xylose, xylose-glucose mixtures and a 5-sugar solution simulating the composition of spent sulphite liquor, and identified conditions for producing butanol-acetone-ethanol solvents in yields above 0.36 g solvent/g sugar utilized, and at the same time over 96% sugar utilization. While these substrates did not contain lignin or the other humic substances present in spent sulphite liquor, our success encouraged us to pursue this approach.

In our other approach, we fermented spent sulphite liquor with the pentose-fermenting ethanol-producing yeast *C. shehatae* [3]. With this yeast, we obtained good xylose utilization at acceptable rates of ethanol production under conditions which appeared to lend themselves to industrial operation, using pure sugars [4]. We now report the application of this process to spent sulphite liquor.

While the fermentation of spent sulphite liquor is long established, SO_2, formic acid and other volatiles and lignin act as inhibitors and should be removed as completely as is practical. However, since the objective of this study is to effect more complete removal of the sugars from the liquor, while the lignin must remain for conversion to vanillin, we are constrained from removing the lignin prior to fermentation. We report our results in removing the volatiles, and at least reducing this source of inhibition.

PRETREATMENT OF SPENT SULPHITE LIQUOR

Samples of spent sulphite liquor were obtained from Ontario Paper Company. Some of these were taken from a storage tank ahead of their SO_2 stripper, which is an indirect steam heat exchanger, and others after their SO_2 stripper. The samples received had pH in the range of 2.4 to 2.9. These samples were fed to a packed tower, with a countercurrent steam flow, while following the pH as control of effectiveness of removal of volatiles. In this way the pH was raised from 2.6 to 4.3 to 4.7. The concentration of toxic substances, namely formic acid, acetic acid, oxalic acid and furfural in the liquor was determined by gas chromatography. The results are shown in Table II. By steam stripping, the total concentration of the steam volatile substances was reduced to less than 6% of their initial concentration.

For butanol fermentation, s.s.l. was steam stripped to pH 4.7. For ethanol fermentation, when this series of experiments began, it was necessary to steam strip to pH 4.5. However, in our ethanol fermentation by *C. shehatae* the yeast was recycled daily. In this way the yeast became acclimatized to s.s.l. At the same time, the cell concentration built up from an original 1 to 2 g/L to 10 to 20 g/L. These later fermentations, with acclimatized yeast at high cell concentrations, required less steam stripping. Satisfactory and rapid fermentation, described below, was obtained with s.s.l. steam-stripped to pH 3.0 to 3.4. Generally, raising the pH by 1.0 to 1.2 units was sufficient to avoid inhibition of fermentation by this acclimatized yeast.

BUTANOL FERMENTATION

Four cultures were used to begin with, *Clostridium butyricum* ATCC 8260; and *Cl. acetobutylicum* strains ATCC 824, NRRL 527 and NRRL 594. The preparation of inocula, the technique of adaptation of these micro-organisms and the fermentation procedures are described in the experimental section below. Although butanol fermentation of pure sugar mixtures simulating s.s.l. by these cultures has shown excellent results [2], and the concentration of toxic substances was minimized by steam stripping to pH 4.7, the s.s.l. was still difficult to ferment in the presence of its lignosulphonate and other phenolic substances. *Cl. acetobutylicum* ATCC 824 and NRRL 527 could not be adapted to s.s.l. under the conditions employed, growing instead into masses of filamentous aggregates. On the other hand, NRRL 594 and *Cl. butyricum* ATCC 8260 were successfully acclimatized to s.s.l.

The results of butanol fermentation are shown in Table III.

While this is the first report of butanol fermentation of s.s.l. containing lignosulphonate (compare [5]), sugar removal in these fermentations is incomplete, and solvent production was substantially less than obtained with a pure sugar mixture simulating s.s.l. [2]. Growth was vigorous, the cells were motile, but no clostridial forms were observed. Various additions to the medium were tried, for example the addition of malt extract, Monot's nutrients [6], molasses, biotin, p-aminobenzoic acid and the antioxidants cysteine, sodium thioglycollate and 9,10-dihydroxyanthracene, but none of these improved sugar consumption or solvent yield. Replenishment of the inoculum after 24 h also offered no improvement. Thus only a partial success can be claimed.

ETHANOL

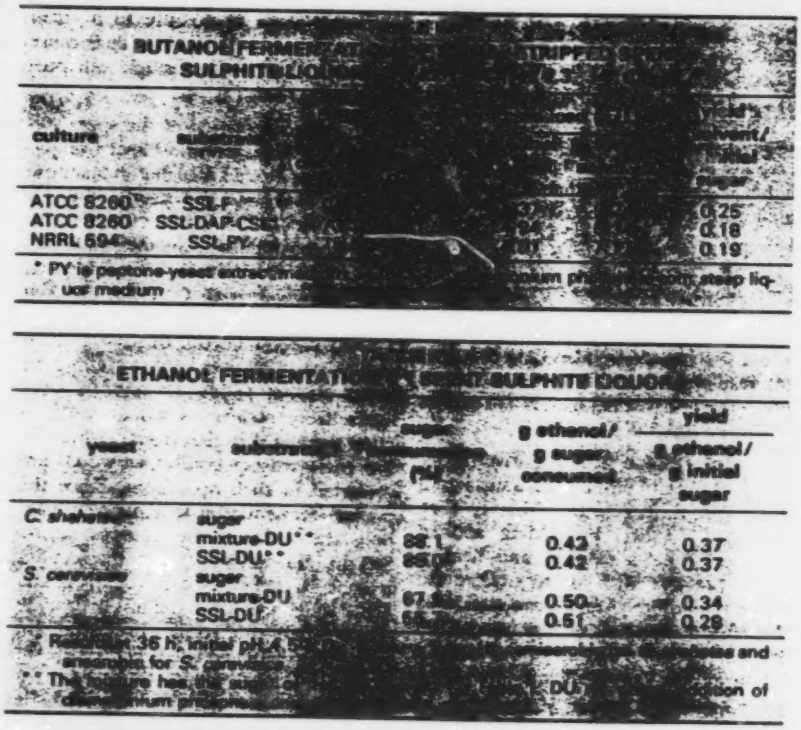
In Table IV are compared the results of fermentation of s.s.l. with a pure sugar mixture simulating s.s.l., of the composition shown in Table I.

From the results it is apparent that *C. shehatae* is more effective at sugar removal from s.s.l. than is *S. cerevisiae*, the improvement in sugar removal being 58.0%; and also in production of alcohol, the improvement being 32.0% in spite of a lower efficiency or yield in terms of ethanol production per gram of sugar consumed, when the added nutrient is DU (diammonium phosphate and urea). The addition of DU is normal practice in alcoholic fermentation of s.s.l.

It is also clear from Table IV that better results are obtained with pure sugars than with s.s.l. by *S. cerevisiae*. There is little doubt that while steam-stripping may remove many toxic substances from s.s.l., inhibitors are still present. The effectiveness of *C. shehatae* in fermenting s.s.l. or the s.s.l.-simulating sugar mixtures was enhanced by the addition of YMP (yeast extract-malt extract-peptone) as nutrient, the results being shown in Table V and in Fig. 1.

While the removal of sugars is enhanced by the addition of the added nutrients of YMP with the pure sugar mixture, reaching almost completion in 36 h, YMP had no effect on sugar removal with s.s.l. It did, however, improve ethanol production by about 10%. Indeed, the yield of 0.464 g ethanol/g sugar consumed must be very close to the theoretical [7].

Figure 1 shows that both sugar consumption and ethanol production



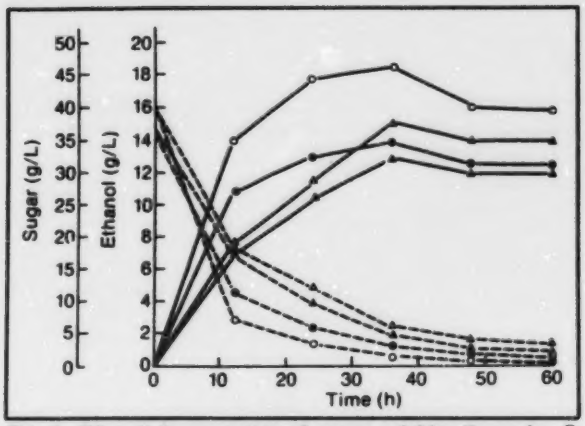


Fig. 1. Ethanol fermentation of spent sulphite liquor by *C. shehatae* ATCC 22984, inoculum 2.5 g/L, semiaerobic.
———— ethanol concentration
– – – – – – – – – – sugar concentration
Δ sugar mixture with DU (diammonium phosphate — urea)
o sugar mixture with YMP (yeast extract-malt extract-peptone)
Δ s.s.l. with DU
• s.s.l. with YMP

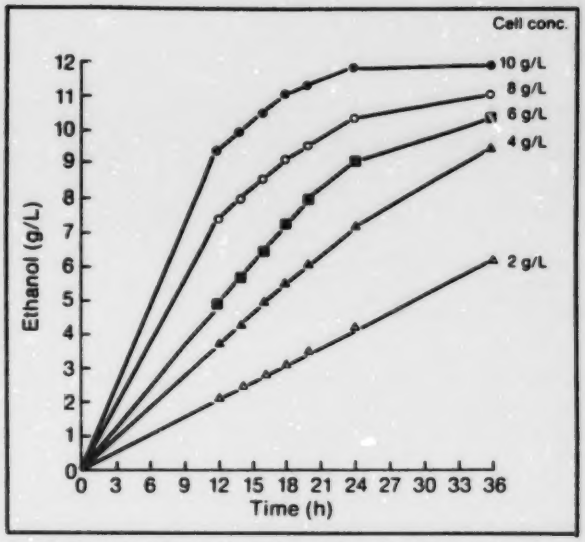


Fig. 2. Fermentation rates as a function of cell concentrations, *Candida shehatae* semiaerobic.

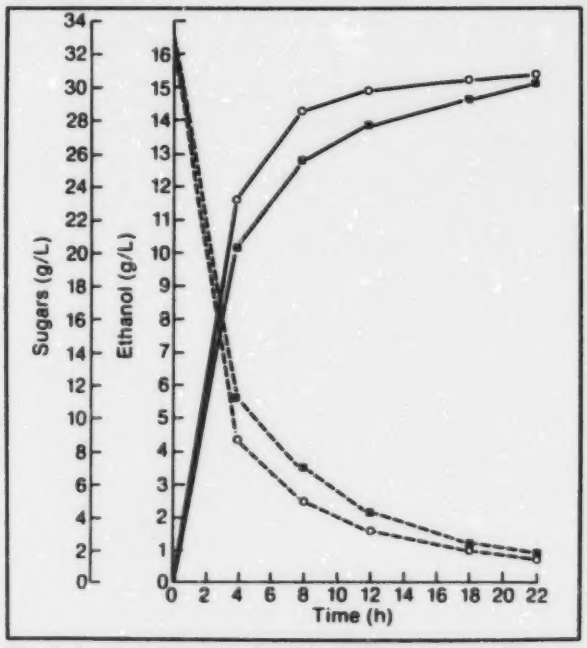


Fig. 3. Fermentation with *Candida shehatae* recycled 60 times in s.s.l., initial pH 2.4, cell concentration 15 g/L.
o — o steam striped to pH 3.4
▪ — ▪ steam stripped to pH 3.0

peaked at 36 h. However, as observed previously in this semiaerobic mode of fermentation [4], ethanol concentration decreased thereafter, indicating consumption by the yeast as well as evaporation losses. Sugar consumption after 36 h was very slight.

The results described above were obtained with fresh cultures of *Candida shehatae* at relatively low cell concentrations up to 2.5 g/L. When cell concentrations were increased, the rate of fermentation increased dramatically, as shown in Fig. 2. When fermenting with 10 g/L yeast, ethanol concentration at 18 h was the same as at 36 h with 8 g/L yeast. In subsequent fermentations, high inocula was adopted as standard procedure, using at least 10 g/L in the fermentation.

These higher cell concentrations were accomplished as a result of total yeast recycling. When yeast was recycled completely from each day's fermentation to the next day's fermentation, the yeast cell concentration increased to about 16 g/L. At such high cell concentrations fermentation was complete in 24 h, as shown in Fig. 3. Efficiency was excellent: 0.48 g ethanol/g sugar consumed, or 0.46 g ethanol/g initial sugar.

C. shehatae recycled 30 times required less steam stripping of the s.s.l. than fresh yeast. Starting with s.s.l. of initial pH 2.9, ethanol production improved as the pH was raised to 4.1. Raising pH to a higher level resulted in less ethanol production, perhaps because of loss of acetic acid or some other volatile substrate.

After recycling 60 times, the requirement for steam stripping was further reduced. The two curves in Fig. 3 represent samples of s.s.l. which initially had pH 2.4 and were steam-stripped to pH 3.4 and 3.0. While ethanol fermentation was complete in 24 h, it may have been economical to terminate the fermentation of the sample steam-stripped to pH 3.4 at 12 h, since not much ethanol was gained thereafter. It would seem that there are benefits to be derived by steam stripping to an increase of 1.0 pH unit. It was also observed that in fermentations of s.s.l. steam stripped to such low pH values the cell concentrations did not increase. Only the better acclimatized cells survived and made use of the sugars. Continued recycling of these acclimatized yeasts may reduce still further the need for steam stripping.

In Table VI are compared the results of ethanol production and sugar removal by *Saccharomyces cerevisiae* and by *Candida shehatae*, in both cases using recycled yeast, the latter recycled 60 times. The results show 33% improvement in ethanol production and 46% improvement in sugar removal.

EXPERIMENTAL

Butanol

Four cultures were used, *Clostridium butyricum* ATCC 8260; *Cl. acetobutylicum* strains ATCC 824, NRRL 527 and

COMPARISON OF FERMENTATION OF [illegible] SULPHITE LIQUOR BY *SACCHAROMYCES CEREVISIAE* AND *CANDIDA SHEHATAE*

	ethanol production			% sugar removal	
	g/L	g ethanol / g initial sugar	relative yield	% initial sugar	relative removal
S. cerevisiae	10.27	0.346	1.0	65.4	1.0
C. shehatae	15.32	0.460	1.33	95.5	1.46

RRL 594. The lyophilized cultures were erminated in reinforced *Clostridium* edium (RCM [8]), followed by sporula-on in 10% corn meal mash at 35°C for 7 ays. The spores were collected by ntrifugation, suspended in distilled ater and stored at 4°C. For inoculation, e spore suspension was heat shocked at °C for 5 min, transferred to corn-liver edium (CL: 6% corn mash, 0.5% Bacto-ver) and incubated at 35°C for 24 h in a ducing atmosphere in an anaerobic ove box [9]. All butanol fermentations ere carried out in 100 mL medium in 250 L shake flasks in an anaerobic atmo-here for 2 or 3 days. Only cultures lapted to spent sulphite liquor (see elow) were used. Inocula were 1 to 2 L.

daptation

Although pure sugar butanol fer-entation has shown excellent results [2] nd the concentration of toxic substances ad been minimized by steam stripping to H 4.7, the s.s.l. was still difficult to rment in the presence of its lignosul-onate and other phenolic substances. daptation was carried out following eat shocking and 10 to 16 h incubation in L, and then transferring to 40% s.s.l., CL r 12 to 18 h, then transferring to 70% .l., 30% CL for 16 to 24 h, the resulting ltures being used as inocula. In the ansfers, the s.s.l. was fortified with ther peptone-yeast extract (PY, pep-ne, 20 g/L, yeast extract 10 g/L) or ammonium phosphate 5 g/L and corn eep liquor 50 g/L (DAP-CSL). Each ansfer was carried out at the height of s production. *Cl. acetobutylicum* ATCC 4 and NRRL 527 could not be adapted s.s.l. under these conditions, growing as asses of filamentous aggregates. Both *. butyricum* ATCC 8260 and *Cl. aceto-tylicum* NRRL 594 were successfully apted to s.s.l.

thanol

Candida shehatae ATCC 22984, ndly provided by Dr. Thomas W. ffries, USDA Forest Products Labora-ry, Madison, Wisconsin, was main-ined on yeast extract-malt agar slants th xylose as the carbon source.

For comparison, *S. cerevisiae* was ed. The strain was obtained from an dustrial alcohol fermentation plant (St. wrence Starch Co. Limited, Mississau-, Ontario).

Adaptation

No adaptation of the micro-organisms to steam stripped s.s.l. was required.

Culture conditions

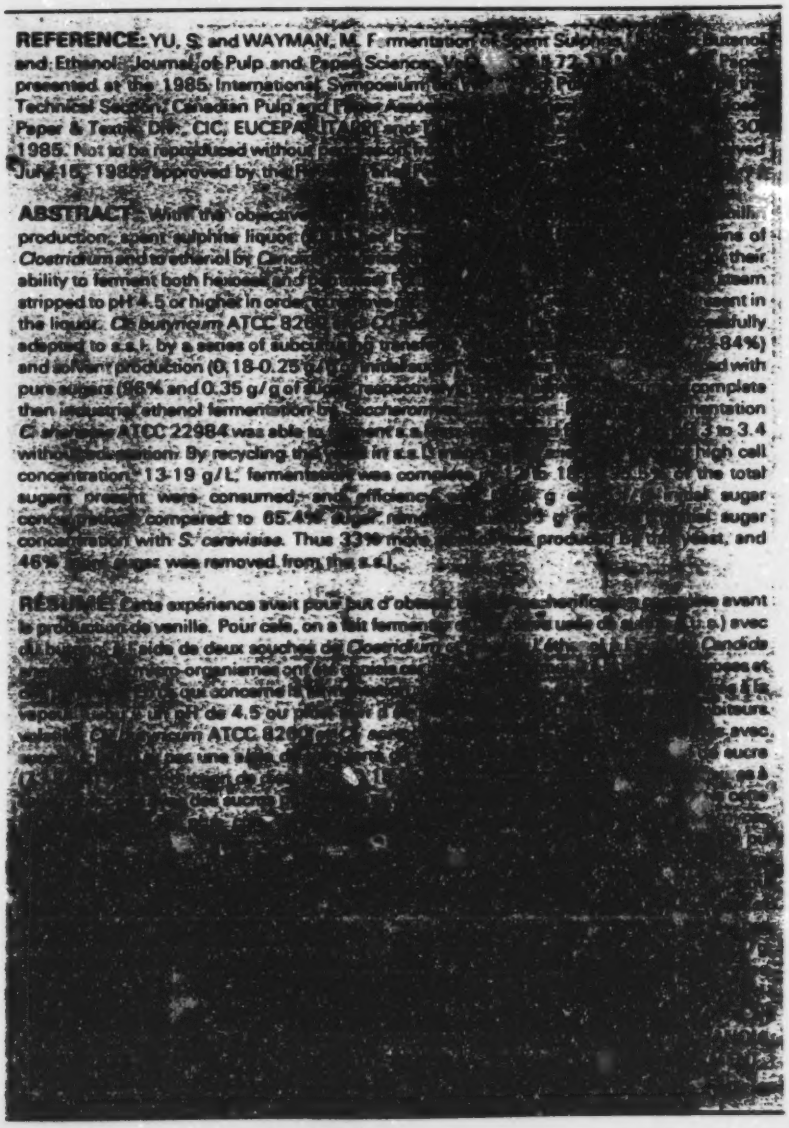
Fermentation was carried out in shake flasks at 30°C, 150 rpm, semiaerobically for *C. shehatae*, anaerobically for *S. cerevisiae*. Cell concentration was 2.5 to 19 g/L after inoculation. Nutrients used were either diammonium phosphate 0.05 g/L, urea 0.1 g/L (DU) or yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L (YMP). The initial pH was 4.5 to 5.5.

Analysis

Solvent and acids were measured by a Hewlett Packard 5700 Gas Chromatograph, the column being packed with 6% FFAP on Poropak Q. Sugar analysis was by DNS [10] and Hewlett Packard HPLC 1090 with a Waters Carbohydrate Analysis Column (Part No. 84038).

CONCLUSIONS

1. Spent sulphite liquor contains many fermentation inhibitors. Some of these may be removed by steam stripping, particularly when this raises the pH to 4.3 or higher. However, some inhibitors remain, resulting in incomplete sugar consumption and lower production of either butanol solvents or ethanol.
2. Two of the butanol-producing micro-organisms studied here are adaptable



REFERENCE: YU, S. and WAYMAN, M. Fermentation of Spent Sulphite [illegible] and Ethanol. Journal of Pulp and Paper Science. [illegible] presented at the 1985 International Symposium [illegible] Technical Section, Canadian Pulp and Paper Association, Paper & Textile Div., CIC, EUCEPA, ITAPP and [illegible] 1985. Not to be reproduced without [illegible] June 15, 1985. Approved by the [illegible]

ABSTRACT – With the objective [illegible] production, spent sulphite liquor [illegible] *Clostridium* and to ethanol by *Candida* [illegible] ability to ferment both hexoses and [illegible] stripped to pH 4.5 or higher in order [illegible] the liquor. *Cl. butyricum* ATCC 826[illegible] adapted to s.s.l. by a series of subcultures [illegible] and solvent production (0.18-0.25 g/[illegible] pure sugars (98% and 0.35 g/g of sugar, respectively). [illegible] then industrial ethanol fermentation by *Saccharomyces* [illegible] *C. shehatae* ATCC 22984 was able to ferment s.s.l. [illegible] without detoxification. By recycling the yeast in s.s.l. [illegible] concentration of 13-19 g/L, fermentation was complete [illegible] the total sugars present were consumed, and efficiency [illegible] g ethanol / g initial sugar compared to 65.4% sugar [illegible] g ethanol / g initial sugar concentration with *S. cerevisiae*. Thus 33% more [illegible] product by yeast, and 46% more sugar was removed from the s.s.l.

RÉSUMÉ – Cette expérience avait pour but d'obtenir [illegible] fermentation [illegible] avant le production de vanille. Pour cela, on a fait fermenter [illegible] (s.s.l.) avec du [illegible] à l'aide de deux souches du *Clostridium* [illegible] *Candida* [illegible] micro-organismes ont [illegible] aptes et [illegible] qui concerne la [illegible] vapeur. [illegible] pH de 4.5 ou plus [illegible] saveurs. [illegible] *Cl. butyricum* ATCC 8260 [illegible] avec une [illegible] sucre [illegible] (7 [illegible] sucre) [illegible] à [illegible] cette [illegible]

to growth on steam-stripped spent sulphite liquor. However while growth is vigorous, solvent production was well below that experienced with pure sugars (0.24 g solvents/g sugar in s.s.l. compared to 0.36 with the pure sugar mixture) and sugar removed was less (73.3% compared to 96.6%). This is, however, better sugar removal than experienced with *S. cerevisiae* (55.7%). If butanol becomes a highly desirable product [11], butanol fermentation is technically feasible.

3. The production of ethanol from spent sulphite liquor by *C. shehatae* is better than by *S. cerevisiae* by a significant margin, 33%, and sugar removal is more complete by 46%.
4. By the use of high cell concentrations, the rate of fermentation to ethanol was increased, fermentation being complete in 24 h when cell concentration was 10 g/L or higher.
5. Steam-stripping from pH 2.4 to pH 3.4 was sufficient to enable rapid, efficient fermentation to take place, 96% of the ultimate ethanol being produced in 12 h, by *C. shehatae* which had been recycled many times.
6. *Candida shehatae* has the potential for being substituted for *Saccharomyces cerevisiae* in industrial ethanol production whenever pentose sugar concentrations are significant.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Thomas W. Jeffries for providing the yeast *Candida shehatae* ATCC 22984, and to thank Ontario Paper Company for the supply of spent sulphite liquor, and for financial aid to one of us (S.Y.). This work was supported in part by the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

1. CRAIG, D., Justification for Pulp and Paper Byproduct Development. Am. Inst. Chem. E. Symp. Series No. 133, Vol. 69, 1-5 (1973).
2. WAYMAN, M. and YU, S., Acetone-Butanol Fermentation of Xylose and Sugar Mixtures. *Biotech. Lett.*, Vol. 7, 255-260 (1985).
3. du PREEZ, J.C. and van der WALT, J.P., Fermentation of D-xylose to Ethanol by a Strain of *Candida shehatae*. *Biotech. Letters*, Vol. 5, 357-362 (1983).
4. WAYMAN, M. and TSUYUKI, S.T., Fermentation of Xylose to Ethanol by *Candida shehatae*. Seventh Symp. on Biotechnol. for Fuels and Chemicals, Gatlinburg, Tennessee, May 14-17, 1985. Biotech. and Bioeng., in press.
5. WILEY, A.J., JOHNSON, M.J., McCOY, E. and PETERSON, W.H., Acetone-butyl Alcohol Fermentation of Sulphite Waste Liquor. *Ind. Eng. Chem.*, Vol. 33, 606-610 (1941).
6. MONOT, F., MARTIN, M.R., PETIT-DEMANGE, H. and GAY, R., Acetone-butanol Fermentation by *Clostridium acetobutyricum* in Synthetic Medium. *Appl. Environ. Microbiol.*, Vol. 44,1318-1324 (1982).
7. GONG, C.-S. and TSAO, G.T., Conversion of D-xylose to Ethanol by Yeasts. Intern. Symp. Ethanol from Biomass, Oct. 13-15, 1982, Winnipeg. Edited by H.E. Duckworth and E.A. Thompson. Royal Society of Canada, Ottawa, pp. 525-555 (1983).
8. LONG, S., JONES, D.T. and WOODS, D.R., Sporulation of *Clostridium acetobutylicum* P262 in a Defined Medium. *Appl. Environm. Microbiol.*, Vol. 45, 1389-1393 (1983).
9. SANTANGELO, J.D., Continuous Butanal Fermentation. M.Sc. thesis, Dept. of Microbiol. and Biochem., The University of Vermont, Burlington, VT, 05405, 1984.
10. MILLER, G.L., Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.*, Vol. 3, 426-428 (1959).
11. WAYMAN, M., HUSTED, G.R., SANTANGELO, J.D., and LEVY, S., Butanol by Extractive Fermentation using Fluorocarbons. Proceedings VI Intern. Symp. Alcohol Fuels Techn., Vol. 2, 239-247 (1984).
12. WAYMAN, M. and BAR-SINAE, Y.L., Separation of Sugars and Lignin in Spent Sulphite Liquor by Hydrolysis and Ultrafiltration. *Tappi*, Vol. 59 (3), 112-114 (1976).

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ETHANOL AND SUGAR TOLERANCE OF CANDIDA SHEHATAE

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SUMMARY

Candida shehatae ATCC 22984 fermented solutions of up to 260 g/L sugars derived by hydrolysis of whole barley. These solutions contained hexose:pentose 70:30, the hexose being mainly glucose from the barley starch and the pentose being mainly xylose. At sugar concentrations of 180 g/L, fermentation was complete in 72 h, yielding 84 g/L ethanol, 0.47 g ethanol/ g sugar. At 260 g/L, fermentation ceased when ethanol concentration reached 100 g/L, but resumed when the ethanol was removed by vacuum distillation, to yield finally 0.50 g ethanol/ g sugar.

INTRODUCTION

Fermentation of xylose to ethanol by strains of Candida shehatae (du Preez and van der Walt, 1983) has attracted widespread interest. This finding is important because hydrolysis of agricultural and forest biomass results in a mixture of hexose and pentose sugars (Wayman, 1984), and the economy of production of liquid fuels is much improved by efficient fermentation of the pentoses.

As part of our studies of liquid fuel production from agricultural and forest biomass (Wayman and Yu, 1985; Yu and Wayman, 1985), we have reported excellent fermentation of xylose solutions by Candida shehatae ATCC 22984 (Wayman and Tsuyuki, 1985).

Ethanolic fermentation of dilute solutions of hexose-pentose mixtures, such as occur in spent sulphite liquor, proceeds rapidly and completely, with very good yields using C. shehatae (Yu and Wayman, 1985). However, sugar solutions of concentrations above about 90 g/L have been slow to ferment, and in many cases, where mixed sugars are present, the hexoses are fermented and the pentoses remain. We are now able to report successful fermentation of sugar solutions of 260 g/L including 78 g/L pentoses.

MATERIALS AND METHODS

Microorganism: The strain of C. shehatae ATCC 22984 employed in these experiments was adapted to wood hydrolysates by semiaerobic fermentation for 24 h, centrifuging, and recycling the yeast. Recycling was repeated 70 times. It was maintained on yeast extract-malt extract agar slants with xylose as the carbon source.

Substrate: Prehydrolysates of whole barley were prepared by St. Lawrence Reactors Limited, Mississauga, Ontario, and further hydrolysed here by an enzyme mixture including cellulase (Meicelase, Meiji Seika), hemicellulase (Miles), cellobiase (Novo), amyloglucanase (Novo), and pectinol (Rohm and Haas, Philadelphia). Xylose was added to the hydrolysate to raise the pentose content from 13.6% to 30%. As controls, mixtures of glucose and xylose in the proportion 70:30 were also used as substrates. All fermentations were performed in shake flasks under semiaerobic conditions as described by Wayman and Tsuyuki, 1985, with an inoculum of 15 g/L of cells. Urea and diammonium phosphate were added as nitrogen sources at 0.1 and 0.2 g/L respectively.

Analysis: Total reducing sugars were determined by the DNS method (Miller, 1959). Glucose was assayed by a YSI Glucose Analyser (Yellow Springs Instruments). Ethanol was determined by G.C.

RESULTS AND DISCUSSION

Results of fermentation of the pure sugar mixture are shown in Table 1 and of the barley hydrolysates in Table 2.

In these experiments, no substrate or product inhibition was observed, except that at the higher concentrations fermentation was slow, and 72 h was required for complete fermentation of 180 g/L sugars. There was no significant difference in fermentation rates or yields between the pure sugar mixture and the barley hydrolysates, although the latter gave slightly higher yields. Average yields of 0.45 g ethanol/ g sugars were realized, giving about 90% of theoretical. Ethanol concentrations of about 80 g/L are quite satisfactory for industrial processing.

When a more concentrated solution of barley hydrolysate, 260 g/L total sugars, hexose:pentose 70:30, was fermented, fermentation was rapid in the first 24 h, but at 48 h, when ethanol concentration reached 100 g/L, fermentation ceased. At this time, residual sugar was 50 g/L, mostly pentose. The fermentation was repeated, but at 24 h, when ethanol concentration was 73 g/L and residual sugar 100 g/L, the ethanol was removed by vacuum distillation at 35°C. Upon adjustment of volume, fermentation resumed, the sugar was essentially all consumed (residual 10 g/L) and total ethanol production reached the equivalent of 125 g/L, or 0.50 g ethanol/ g sugar consumed. These results are shown in Fig. 1.

TABLE 1 Fermentation of pure sugars, glucose:xylose 70:30, by *Candida shehatae*

Initial Sugar Conc. g/L	Residual Sugars g/L	Fermentation Time h	Ethanol Concentration g/L	Ethanol Yield g ethanol/ g sugar
49	5	24	19	0.43
98	0	48	40	0.41
144	10	48	59	0.44
183	7	72	78	0.44

TABLE 2 Fermentation of barley hydrolysates with pentose concentration raised to 30% of total sugars, by *Candida shehatae*

Initial Sugar Conc. g/L	Residual Sugars g/L	Fermentation Time h	Ethanol Concentration g/L	Ethanol Yield g ethanol/ g sugar
46	0	24	21	0.46
100	0	24	43	0.43
160	0	72	73	0.45
180	6	72	84	0.48

These experiments have shown that *Candida shehatae* ATCC 22984 will tolerate and ferment mixed hexose-pentose sugar solutions of concentrations up to 180 g/L without inhibition other than a slowing of fermentation rate at the higher concentrations, with yields of about 0.45 g ethanol/ g sugar. Ethanol concentrations under these conditions were about 80 g/L, and the yeast is able to tolerate such high concentrations. However, when the ethanol concentration reached 100 g/L, fermentation of pentose sugars ceased. The yeast can tolerate, apparently, quite high ethanol concentrations, but not for pentose fermentation. However, when the ethanol was removed by low temperature vacuum distillation, fermentation of pentose resumed, and very good yields of ethanol resulted.

This work suggests that for the fermentation of very high sugar concentrations such as may be derived from the hydrolysis of whole grains, a vacuum-assisted fermentation may be the process of choice.

ACKNOWLEDGEMENTS

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REFERENCES

du Preez, J.C. and van der Walt, J.P. 1983. Biotechn. Letts. 5:357-362.
Miller, G.L. 1959. Anal. Chem. 3:426-428.
Wayman, M., Tallevi, A. and Winsborrow, B. 1984. BIOMASS 6:183-191.
Wayman, M. and Tsuyuki, S. 1985. VII Intern. Symp. on Biomass for Fuels and Chemicals, Gatlinburg, Tennessee; Biotechn. and Bioeng., in press.
Wayman, M. and Yu, S. 1985. Biotechn. Letts. 7:255-260.
Yu, S. and Wayman, M. 1985. J. Pulp and Paper Sci., in press.

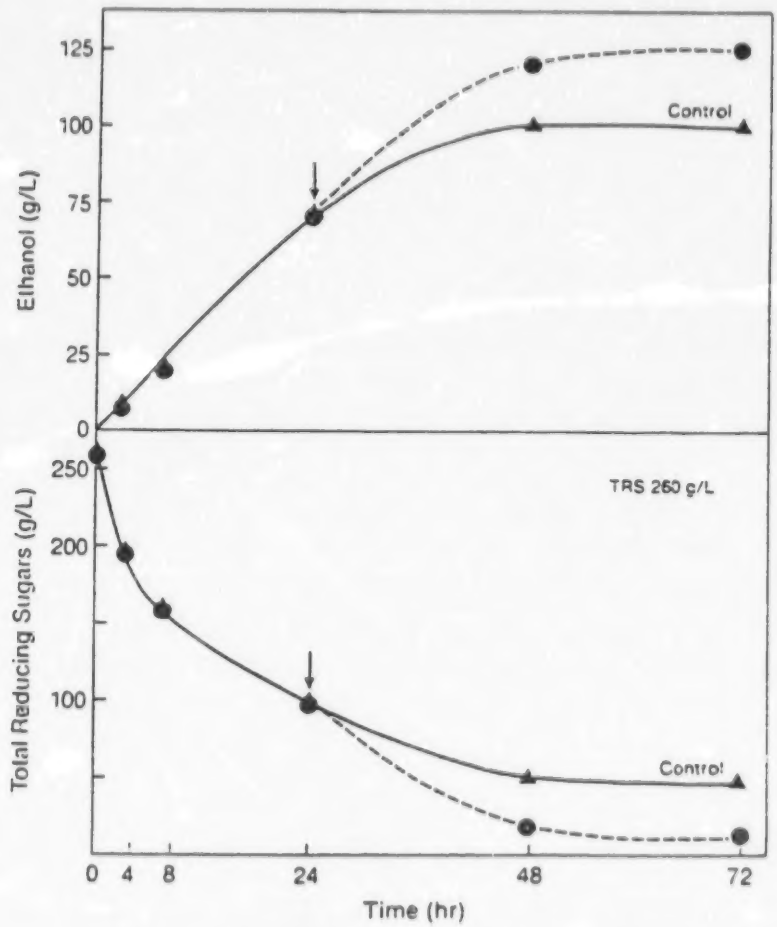

Fig. 1 Production of ethanol from barley hydrolysate containing 30% pentose. Arrow refers to the removal of ethanol by vacuum distillation.

FERMENTATION TO ETHANOL OF PENTOSE-CONTAINING SPENT SULPHITE LIQUOR

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INTRODUCTION

The purpose of our research was to improve the fermentation of spent sulphite liquor (SSL), ~~particularly that produced by a large Canadian sulphite pulp and paper mill which ferments the SSL to ethanol,~~ and uses the alcohol plant effluent as a source of lignin for vanillin manufacture[1]. In present practice in this and in many other mills, the SSL is fermented with bakers' yeast, Saccharomyces cerevisiae, which is a reasonably efficient converter of glucose in the SSL, and perhaps also mannose and galactose, these three being the hexose sugars present in wood. Furthermore, this yeast is fairly resistant to many of the inhibitors present in SSL, and it is sufficiently robust to be well suited to industrial operations. In other words,it produces ethanol in yields which have become acceptable, and does not cause trouble. However, SSL contains not only hexose sugars but also pentose sugars, and in addition to these monomers, also contains oligomers which may constitute as much as one-third of the carbohydrates in solution[2]. We have, in the research reported here, investigated the use of a yeast, Candida shehatae ATCC 22984 which ferments both pentoses and hexoses[3] for ethanolic fermentation of SSL, with highly favorable results in terms of increased alcohol production and improved sugar removal. We also report briefly on additional benefits obtained when this fermentation followed enzyme saccharification of the oligomers present in the SSL. Our major finding is that this yeast is able to ferment both hexoses and pentoses simultaneously, efficiently and rapidly.

EXPERIMENTAL

Candida shehatae ATCC 22984 was originally provided by Dr. Thomas W. Jeffries, USDA Forest Products Laboratory, Madison, Wisconsin. Saccharomyces

cerevisiae and spent sulphite liquor was provided by Ontario Paper Company, ~~Thorold, Ontario~~. Barley hydrolysate was provided by St. Lawrence Reactors, Mississauga, Ontario. Fermentations were carried out in shake flasks at 30°C, 150 rpm, anaerobically for S. cerevisiae, anaerobically or semiaerobically for C. shehatae; and also in 500 mL and 3 L New Brunswick fermenters. Nutrients used were diammonium phosphate 0.05 g/L and urea, 0.1 g/L. Sugar concentrations were analysed with a Hewlett Packard (HPLC) HP1090, with a Waters carbohydrate analysis column. Dissolved oxygen was measured using Chemtrix Type 30 oxygen meter and ethanol by gas chromatography. Cell concentrations of yeasts were determined by their optical density (OD) at 600 nm.

RESULTS AND DISCUSSION

Removal of Inhibitors

The composition of the SSL used in these studies is shown in Table I. In addition to lignosulphonic acids, the SSL contained about 31 g/L of monomeric sugars, of which almost half was mannose, the principal non-glucose sugar of softwoods. Altogether, 73% of the monomeric sugars were hexoses, and 27% were pentoses. Also, there is a considerable amount of oligomeric sugars probably about 12 g/L[1,2]. There are also present formic acid, acetic acid, furfural and acid-soluble lignin fragments. These all act as inhibitors to fermentation.

The volatile inhibitors were removed by steam stripping, the results being shown in Table II. The rise in pH was used as the indicator of removal of toxic substances[4]. As the table shows, raising pH from 2.2 to 3.0 by direct

steam stripping removed 76% of these inhibitors, while further steam stripping to pH 3.4 removed 88%. This had a marked effect on ethanol production, as shown in Figure 1. With Candida shehatae, ethanol production improved markedly as pH was raised by steam stripping from 2.9 to 4.1. Further steam stripping, however, reduces alcohol production. This latter effect is probably due to acetic acid, which is an inhibitor at high concentrations but a fermentation substrate at low concentrations.

Cell Concentration

The amount of cells used for inoculum proved to have a large effect on the rate of ethanolic fermentation, as shown in Figure 2. As cell concentrations were increased from 2 g/L to 10 g/L fermentation was completed in 24 h compared to about 72 h. After we obtained these results, we adopted a cell concentration in the fermenting SSL of about 15-19 g/L as our standard. Further increases in cell concentration did not appear to confer further benefits.

Cell Recycling

Of great importance in conferring resistance to inhibitors and improving the rate of fermentation was our practice of recycling the yeast from each fermentation as the inoculum for the next fermentation. This required centrifuging for recovery of the yeast. This yeast is somewhat fragile, and is also susceptible to autolysis. In our practice we reduced centrifuge speeds to about one-fifth of what we normally use with bakers' yeast, and we also carried out the centrifuging as soon as possible after the fermentation was complete, that is within 24 h of start of fermentation.

One important effect of recycling was to reduce the amount of steam stripping required for rapid, complete fermentation. We concluded from our results that a rise in pH of 1.0 by steam stripping was quite enough for successful fermentation by our recycled yeast.

We have now some evidence that the recycled yeast has important differences from the parent strain, in terms of ability to withstand inhibitors, and also of ability to ferment pentoses. The necessary enzymes for efficient fermentation of wood sugars and mechanisms which confer resistance to inhibitors appear to have been induced in, and to have become part of, the yeast. We have labelled this new, recycled yeast with the title Candida shehatae (R). A comparison of fermentation rates by the parent strain and the recycled strain is shown in Figure 3.

Air Requirement during Fermentation

Common bakers' yeast requires an anaerobic atmosphere for ethanolic fermentation. With ordinary yeast, the presence of oxygen leads to the consumption of ethanol and the growth of the yeast at the expense of ethanol production. A small amount of oxygen is generally beneficial for maintenance and vigorous performance of the yeast, but it is customary to refer to this as an anaerobic fermentation. Candida shehatae (R), in common with other pentose-fermenting yeasts, does not do well in an anaerobic environment. Some air is required for pentose fermentation. However, too much air leads to the consumption of the ethanol by the yeast. The best conditions are a compromise, maximum ethanol production and pentose-consumption requiring the availability of oxygen at a strictly controlled rate, a condition we refer to as "semiaerobic".

The course of a typical semiaerobic fermentation of SSL with Candida shehatae (R) is shown in Figure 4. Dissolved oxygen is consumed rapidly in the first few hours, reaching a minimum at 4 hours, and then slowly increases to saturation in the next 10 hours. At that same time the cell concentration has reached its maximum at about 8 hours, having increased from 15 g/L to 18 g/L. Sugar consumption and alcohol concentration increase rapidly in the first 8 hours, and are essentially complete at 12 h. At 12 h, ethanol formation from the SSL by this yeast is highly efficient, 0.50 g ethanol/g sugar consumed, very close to the theoretical limit.

Some experiments were carried out in a 3 L fermenter to determine the air volume required for best fermentation of pentose-hexose mixtures. The best fermentation was obtained when air passed through the fermenting mixture at the rate of 2.2 volumes per volume of medium per hour for the first 12 hours, and then the rate was reduced to 0.8 volumes per hour for the rest of the fermentation period. At high cell concentration such as this, fermentation is quite rapid, being essentially complete in 8 to 18 h, depending upon conditions. When the same SSL is fermented anaerobically, fermentation takes place, but more slowly, as shown in Figure 5. In the run shown, in 24 h 10.5 g/L ethanol were produced, with sugar consumption of 25 g/L, that is with an efficiency of 0.42 g ethanol/g sugar consumed. Furthermore, only 83% of the initial sugar was consumed, whereas in the semiaerobic fermentation shown in Figure 4, 95% of the initial sugar was consumed. Thus the semiaerobic mode of fermentation is more rapid and more efficient both in alcohol production and in sugar consumption. In later runs, with high cell concentrations of the (R) strain recycled many times, many semiaerobic fermentations were complete in 6 to 8 hours.

Comparison of Candida shehatae (R) and Saccharomyces cerevisiae

Table III compares the productivity and efficiency of Candida shehatae (R) and Saccharomyces cerevisiae. The C. shehatae fermentations were carried out both semiaerobically and anaerobically. Fermentation time for the S. cerevisiae anaerobic and the C. shehatae semiaerobic were both 18 hours, whereas the anaerobic C. shehatae fermentation required 48 hours. The productivity of the S. cerevisiae measured as g ethanol/g sugar consumed was higher than that of the C. shehatae, but the amount of sugar consumed and the amount of ethanol produced were much less. The semiaerobic C. shehatae (R) fermentation produced 33% more ethanol than the S. cerevisiae. In terms of sugar removal, the S. cerevisiae was much poorer, the C. shehatae semiaerobic removing 46% more sugar than the S. cerevisiae.

The consumption of the individual monomeric sugars in the SSL is shown in Table IV. As expected S. cerevisiae utilizes fully glucose and mannose, and about half the galactose present in the SSL, while the xylose and arabinose remain unfermented. The C. shehatae (R) ferments all the xylose, glucose and mannose, while only 42% of the arabinose is utilized. There is some evidence that the arabinose is utilized preferentially for energy and cell growth, but is not fermented to ethanol. Total sugar utilization by the C. shehatae (R) is 95%, compared to only 65% by the S. cerevisiae.

Sugar and Ethanol Tolerance

For industrial utilization, especially in the fermentation of hexose-pentose mixtures more concentrated than those occurring in SSL, it is important to establish the limits of tolerance of this new yeast to ethanol and to high sugar concentrations. This was studied using pure sugar mixtures

containing glucose:xylose in the ratio of 70:30; and also with hydrolysates of whole barley fortified with xylose to the same glucose:xylose ratio. The results, shown in Table V, indicate that there is no difficulty in fermenting sugar solutions up to 180 g/L concentration, although while up to 49 g/L required 24 hours for complete fermentation, higher concentrations were slower, the 180 g/L solution requiring 72 h.

When still higher concentrations of sugars were fermented, ethanol became an inhibitor when its concentration reached about 100 g/L. At this point fermentation ceased. However, when the ethanol was removed by low temperature vacuum distillation, fermentation resumed. Solutions of 260 g/L mixed sugars (barley hydrolysate fortified with xylose to glucose: xylose = 70:30) were fermented with very high productivity, about 0.50 g ethanol/g sugar consumed, as shown in Figure 6.

Byproducts

In fermentation of SSL, xylitol, sorbitol and mannitol are formed in small amounts as byproducts. This is shown in Table VI. Xylitol first showed up as a byproduct of semiaerobic fermentation by C. shehatae (R) after 16 recycles, whereas it did not appear when the same fermentation was carried out anaerobically. Sorbitol and mannitol were formed in the early recycles of both semiaerobic and anaerobic fermentations, but by the 16th recycle, they no longer appeared in the semiaerobic fermentations, although they were present in the anaerobic fermentation. Thus xylitol does not appear in the finished anaerobic fermentation, but the hexitols are present, whereas in the semiaerobic fermentations the hexitols do not accumulate on repeated recycling, while the xylitol does then appear as a byproduct.

Enzyme-assisted Fermentation of SSL

Figure 7 compares fermentation of SSL by C. shehatae (R) with and without prior enzyme hydrolysis of oligomers. Spent sulphite liquor was hydrolysed at 50°C for 18 hours by an enzyme mixture including cellulase (Meicelase, Meiji Seika), hemicellulase (Miles), and cellobiase (Novo). About 20% more ethanol was obtained when enzyme saccharification preceded fermentation. This is a preliminary result, which suggests that, while the use of C. shehatae (R) confers many tangible benefits on fermentation of SLL, further benefits may be obtained by hydrolysis of the oligomeric sugars present in the liquor.

CONCLUSIONS

1. Candida shehatae (R) ferments SSL more completely than the common Saccharomyces cerevisiae, resulting in 33% increase in alcohol production and in 46% increase in sugar removal.
2. Fermentation is rapid and efficient when the SSL is steam stripped to raise pH by at least 1 unit, when using high cell concentrations of 10 g/L and above, and when the yeast is recycled through successive fermentations many times. A small, controlled amount of air is necessary in these fermentations to ensure good fermentation of pentoses.
3. C. shehatae (R) is tolerant of sugar concentrations to at least 250 g/L and of alcohol concentrations to about 100 g/L. Complete and efficient fermentation of such highly concentrated sugar solutions required low temperature vacuum distillation of the alcohol.
4. Under some circumstances, xylitol, mannitol and sorbitol are formed in low concentrations as byproducts of fermentation of SSL.

5. Enzyme saccharification of the sugar oligomers in SSL results in higher alcohol yields.
6. C. shehatae (R) is able to ferment both hexoses and pentoses simultaneously, efficiently and rapidly. Present indications are that it is well suited to industrial operations wherever hexoses and pentoses are both to be fermented to ethanol, for example, in wood hydrolysates.

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REFERENCES

1. D. Craig, Am. Inst. Chem. E. Symp. Series No. 133, 69, 1-5 (1973).

2. M. Wayman and Y.L. Bar-Sinae, Tappi, 59(3), 112-114 (1976).

3. J.C. du Preez and J.P. van der Walt, Biotech. Letters, 5, 357-362 (1983).

4. S. Yu, Ph.D. Thesis, University of Toronto, Toronto, Canada, 1986.

Table I. Composition of Spent Sulphite Liquor.

	SSL of Jan. 18, 1985	SSL of May 16, 1985
pH	2.6	2.2
	g/L	
Xylose	6.51	7.83
Arabinose	1.25	1.65
Mannose	13.58	16.16
Glucose	2.86	3.28
Galactose	5.34	4.37
Total Sugars*	29.54	33.29
Lignosulphonic acids as lignin	67.69	71.18

* In addition to these monomeric sugars, there is a considerable content of oligomeric carbohydrates, which may amount to 30% of the total carbohydrate content (1).

Table II. Comparison of Direct and Indirect Steam Stripping.

	Original SSL (May 16, 1985)	Indirect Steam Stripping (by OPC)	Direct Steam Stripping			
			to pH 3.0	3.4	4.0	4.3
pH	2.2	2.6	3.0	3.4	4.0	4.3
relative concentration of toxic substances	1.00	0.87	0.24	0.12	0.09	0.05

Table III. Comparison of C. shehatae and S. cerevisiae in Fermenting Spent Sulphite Liquor.

Yeast	Aeration condition	Fermentation time (h)	Ethanol production			Sugar removal	
			g ethanol/ g sugar consumed	g ethanol/ g sugar initial	relative yield	% initial sugar	relative removal
S. cerevisiae	anaerobic	18	0.530	0.346	1.0	65.4	1.0
C. shehatae	semiaerobic	18	0.482	0.460	1.33	95.5	1.46
C. shehatae	anaerobic	48	0.420	0.373	1.08	88.8	1.36

Table IV. Comparison of Sugar Utilization in Fermentation of Spent Sulphite Liquor by *S. cerevisiae* and by *C. shehatae*.

sugar	by *S. cerevisiae*			by *C. shehatae*		
	initial g/L	remained g/L	utilization % of initial	initial g/L	remained g/L	utilization % of initial
xylose	6.78	6.83	0	7.83	—	100
arabinose	1.42	1.32	0	1.65	0.95	42.4
mannose	13.88	—	100	16.16	—	100
glucose	3.06	—	100	3.28	—	100
galactose	4.58	2.12	53.7	4.37	0.54	87.6
total	29.72	10.27	65.4	33.29	1.49	95.5

Table V. Sugar and Ethanol Tolerance of C. shehatae.

Initial Sugar Conc. g/L	Residual Sugars g/L	Fermentation Time h	Ethanol Concentration g/L	Ethanol Yield g ethanol/ g sugar consumed
A. Fermentation of pure sugars, glucose:xylose 70:30, by Candida shehatae				
49	5	24	19	0.43
90	0	48	40	0.41
144	10	48	59	0.44
183	7	72	78	0.44
B. Fermentation of barley hydrolysates with pentose concentration raised to 30% of total sugars, by Candida shehatae				
46	0	24	21	0.46
100	0	24	43	0.43
160	0	72	73	0.45
180	6	72	84	0.48

Table VI. Fermentation Byproducts.

No. of Cycles*	Xylitol**	Sorbitol and Mannitol**
3 Sem.	-	+
3 An.	-	+
5 Sem.	-	+
5 An.	-	+
16 Sem.	+	-
16 An.	-	+
60 Sem.	+	-

* Sem. = Semiaerobic
An. = Anerobic

** + = significant
- = not significant

LEGENDS TO THE FIGURES

Figure 1. Effects of steam stripping on ethanol production; C. shehatae in spent sulphite liquor, initial pH 2.2.

Figure 2. Fermentation rates as a function of cell concentrations; C. shehatae, semiaerobic.

Figure 3. Ethanol fermentation by recycled cells of C. shehatae.

Figure 4. Typical ethanol fermentation of spent sulphite liquid by C. shehatae recycled 60 times, 3 L fermenter, 30°C, semiaerobic.

Figure 5. Anaerobic fermentation of spent sulphite liquor by C. shehatae, cell concentration 10 g/L, 30°C.

Figure 6. Production of ethanol from high concentration whole barley hydrolysates containing 30% pentose. Arrow refers to removal of ethanol by vacuum distillation.

Figure 7. Ethanol production by C. shehatae from spent sulphite liquor with and without enzyme saccharification.

(—x—x) without enzyme saccharification

(•—•) with enzyme saccharification

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Adaptation of *Candida shehatae* and *Pichia stipitis* to wood hydrolysates for increased ethanol production

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SUMMARY

Candida shehatae ATCC 22984 and Pichia stipitis CBS 5776 were tested for ethanol production from xylose, glucose-xylose mixtures, and aspen wood total hydrolysates. Adaptation of these yeasts to wood hydrolysate solutions by recycling resulted in improved substrate utilization and ethanol production. Compared to the non-adapted cultures, recycled C. shehatae and P. stipitis in aspen hydrolysate increased g ethanol/g sugar consumed from 0.39 and 0.41 to 0.45 and 0.47; while ethanol production from a 70:30 glucose-xylose solution (total sugars 140 g/L) was 45 g/L in 24 h and 60 g/L in 72 h with the adapted yeasts compared to 15 g/L and 28 g/L in the same times with the parent strains.

INTRODUCTION

Recent reports on the ability of Candida shehatae and Pichia stipitis to ferment xylose efficiently to ethanol have attracted widespread attention and have prompted investigation into the use of lignocellulosic and wood hydrolysates as fermentable substrate (du Preez and van der Walt, 1983; Slininger et al, 1985; Wayman and Tsuyuki, 1985; Yu and Wayman, 1986). The economic feasibility of using hydrolysed biomass in industrial fermentation processes will depend on the successful and efficient fermentation of pentose sugars and the tolerance of these yeasts to toxic substances, eg. lignins, phenols, wood extractives and metal ions from equipment corrosion (Azhar et al, 1982; Gong and Tsao, 1983). Pretreatments by steam stripping, activated carbon and use of ion exchange resins have been shown to alleviate the inhibition effects and give better ethanol yields from wood-derived hemicellulose hydrolysates (Gong and Tsao, 1983; Fein et al, 1984). Alternatively yeast cultures adapted to these hydrolysates by repeated recycling could also be used to overcome such inhibitions and limitations. Improved ethanol yields from D-xylose utilizing recycled cells of Pachysolen tannophilus (Maleszka et al, 1981) and through strain adaptation of Candida tropicalis (Fein et al,

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1984) have been reported. Recently we have shown the potential use of recycled C. shehatae for industrial application in desugaring of sulfite spent liquor with elevated levels of ethanol (Yu and Wayman, 1986). This paper reports on the application of recycled cells of C. shehatae and Pichia stipitis as a means of improving efficiency in xylose, glucose-xylose, and aspen wood hydrolysate fermentations.

MATERIALS AND METHODS

Media: Inoculation medium was prepared in 250 mL shake flasks with 100 mL medium containing 40 g/L xylose, 5 g/L peptone, 3 g/L malt extract and 3 g/L yeast extract at pH 5.8. The xylose fermentation medium was similar to the inoculation medium, except that 5 g/L $(NH_4)_2SO_4$ was substituted for peptone and the xylose concentration was 105 g/L. Aspen wood hydrolysate was prepared as follows: Aspen wood chips were prehydrolysed using 3% SO_2 (w/w) as the hydrolysing catalyst at 150°C for 20 min (Wayman et al, 1984). The pH of the hydrolysates was raised to 4.8 with $Ca(OH)_2$ and further hydrolysed for 18 hours at 50°C with an enzyme mixture that included cellulase (Meicelase, Meiji Seika), hemicellulase (Miles) and cellobiase (Novo). The resulting hydrolysate, containing 70% hexoses and 30% pentoses totalling 92 g/L served, with the addition of 0.2 g/L urea and 0.1 g/L diammonium phosphate, as the medium for recycling and for the fermentation reported in Table 1. For comparison, glucose-xylose medium contained 98 g/L D-glucose, 42 g/L D-xylose, 0.2 g/L urea and 0.1 g/L diammonium phosphate.

Microorganisms: Candida shehatae ATCC 22984 was obtained from the American Type Culture Collection, and Pichia stipitis CBS 5776 was obtained from the Centraalbureau voor Schimmelcultures. They were maintained on yeast extract-malt extract (YM) slants with xylose as the carbon source. The parent strains were adapted to wood hydrolysate medium by semiaerobic cultivation for 24 hours, centrifuging and recycling the yeasts to fresh wood hydrolysate medium. The C. shehatae was recycled 72 times and the P. stipitis 12 times. These adapted strains are referred to here as C. shehatae (R) and P. stipitis (R). They were maintained on YM slants with xylose as the carbon source.

Culture conditions: Inoculum was prepared in inoculation medium, the flasks being inoculated and incubated on a rotary shaker at 150 rpm for 3 days at 30°C. Cells were harvested by centrifugation, washed twice with saline and the resulting pellet inoculated into the fermentation medium. All batch fermentations were performed at pH 5.5 under semiaerobic conditions at 30°C as described (Wayman and Tsuyuki, 1985), with 8.5 g cells (dry weight) per litre.

Analysis: Total reducing sugars were determined by the DNS method (Miller, 1959) using the appropriate factors for glucose, xylose and mixtures. Glucose was assayed by YSI Glucose Analyzer (Yellow Springs Instruments). Ethanol was determined by gas chromatography.

RESULTS AND DISCUSSION

Results of the fermentation of D-xylose and aspen wood hydrolysates are shown in Table 1, and of the

Table 1 Fermentation of D-xylose, and aspen wood hydrolysates by adapted Candida shehatae and Pichia stipitis

Substrate	Strain	% Substrate utilized	Ethanol g/L	Yield g ethanol/ g sugar utilized
xylose[a]	C. shehatae ATCC 22984	79	30	0.37
	P. stipitis CBS 5776	73	34	0.44
	C. shehatae (R)	95	42	0.42
	P. stipitis (R)	88	40.3	0.44
aspen wood hydrolysate[b]	C. shehatae ATCC 22984	71	25	0.39
	P. stipitis CBS 5776	78	29	0.41
	C. shehatae (R)	93	38.5	0.45
	P. stipitis (R)	95	41	0.47

a Initial xylose was 105 g/L; results were taken at 96 hours.
b Initial total reducing sugars were 92 g/L; results were taken at 48 hours.

glucose-xylose medium in Figure 1.

Fermentation of xylose with recycled C. shehatae and P. stipitis showed a dramatic improvement in substrate utilization and, at the same time, resulted in higher concentration of ethanol. With unadapted cultures, fermentations were far from complete at the end of 72 hours, when the fermentations were terminated. Both the recycled strains showed about 30% improvement in substrate utilization and a marked increase in ethanol production of 40% in the case of C. shehatae (R) and 18% with P. stipitis (R).

Although lower sugar concentrations are present in the aspen wood hydrolysate medium, attempts to ferment these hydrolysates with unadapted strains always resulted in slow and incomplete fermentation. Adaptation produced better results (Table 1). With P. stipitis (R), almost complete substrate utilization was achieved in 48 h with ethanol yields of 0.47 g ethanol/g sugar consumed, which is 94% of the theoretical ethanol yield of 0.50 g/g sugar consumed for this sugar mixture. Improvement in ethanol yields was also realized with C. shehatae (R). These results were confirmed with the glucose-xylose sugar mixture (Fig. 1). The results clearly demonstrated the significant potential for using recycled cultures for improving rates and fermentation yields. Fermentation was quite rapid using the recycled cultures of P. stipitis (R) and C. shehatae (R). The adapted cultures accumulated almost twice the

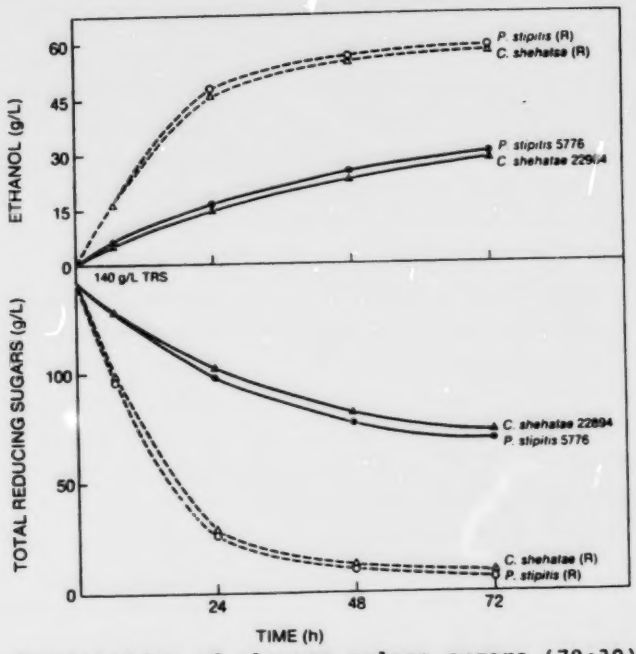

Figure 1. Fermentation of glucose-xylose sugars (70:30) by Candida shehatae ATCC 22984 and Pichia stipitis CBS 5776 and by the same cultures recycled on wood hydrolysate, designated (R).

concentration of ethanol in 72 h compared to the unadapted cultures. The initial, slower fermentation rate with the latter cultures may be due to lack of tolerance of high substrate concentrations (Wayman and Parekh 1985).

These results clearly imply that low conversion yields and ethanol concentrations found with unadapted fresh cultures could easily be overcome by "strain improvement" using the cell recycling technique. How these high ethanol producing yeast strains are generated upon repeated recycling is not known. It may be that recycling results in selection of an adapted strain with inducement of the enzymes required for efficient pentose fermentation. Studies of the genetic changes associated with recycling and adaptation to wood hydrolysates including the isoenzymes profiles and possible metabolic pathways followed by these yeasts, will be reported shortly.

CONCLUSION

The study indicates that strain improvement through recycling represents a useful technique and is an important consideration in efforts to develop industrial scale fermentation procedures for lignocellulose hydrolysates.

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REFERENCES

Azhar, A.F., Bery, M.K., Colcord, A.R. and Roberts, R.S. (1982) Development of a yeast strain for the efficient ethanol fermentation of wood acid hydrolysate. Dev Ind Microbiol 23:351-360.

du Preez, J.C. and van der Walt, J.P. (1983) Fermentation of D-xylose to ethanol by a strain of Candida shehatae. Biotechnol Lett 5:357-362.

Fein, J.E., Tallim, R.S. and Lawford, R.G. (1984) Evaluation of D-xylose fermenting yeasts for utilization of a wood-derived hemicellulose hydrolysate. Can J Microbiol 30:682-690.

Gong, C.S. and Tsao, G.T. (1983) Conversion of D-xylose to ethanol by yeasts. In: International Symposium on Ethanol from Biomass, Oct. 13-15, 1982 Winnipeg, Edited by H.E. Duckworth and E.A. Thompson, Royal Society of Canada, Ottawa 525-555.

Maleszka, R., Veliky, A.I. and Schneider, H. (1981) Enhanced rate of ethanol production from D-xylose using recycled or immobilized cells of Pachysolen tannophilus. Biotechnol Lett 3:415-420.

Miller, G.L. (1959) Use of Dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 3:426-428.

Slinginger, P.J., Bothast, R.J., Okos, J.R. and Ladish, M.R. (1985) Comparative evaluation of ethanol production by xylose-fermenting yeasts presented high xylose concentrations. Biotechnol Lett 7:431-436.

Wayman, M. and Parekh, S. (1985) Ethanol and sugar tolerance of Candida shehatae. Biotechnol Lett 7:909-912.

Wayman, M. and Tallevi, A. and Winsborrow, B. (1984) Hydrolysis of biomass by sulphur dioxide. Biomass 6:183-191.

Wayman, M. and Tsuyuki, S. (1985) Fermentation of xylose to ethanol by Candida shehatae. Biotechnol and Bioeng, Symp. No. 15, 167-177.

Yu, S. and Wayman, M. (1986) Fermentation of spent sulphite liquor to butanol and ethanol. J Pulp and Paper Sci 12(3) J 72-77.

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FERMENTATION OF CELLOBIOSE AND WOOD SUGARS TO ETHANOL BY CANDIDA SHEHATAE AND PICHIA STIPITIS

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SUMMARY: Three pentose fermenting yeast strains of Candida shehatae and three of Pichia stipitis were examined for their ability to produce ethanol from cellobiose and from sugars liberated by hydrolysis of lignocellulosic biomass. All of the Pichia strains tested produced some ethanol; P. stipitis CBS 5776 gave the highest yield: 10.3 g/L on complete fermentation of 25 g/L cellobiose within 48 hours. This yeast also produced considerably more ethanol from the wood sugar mixture.

INTRODUCTION

Conversion of cellulosic material into ethanol is accomplished by the pretreatment of the lignocellulose followed by enzymatic or acidic saccharification and fermentation of the sugars so produced to ethanol (Wayman et al., 1984). Enzymatic saccharification using cellulases is promising, since the process is non-polluting and total saccharification can be achieved. However, conversion times are much longer than with acid hydrolysis and probably require recycling of the enzyme for process economy (Goldstein, 1981). Cellulases are also deficient in β-glucosidase, resulting in an accumulation of the end-product inhibitor, the disaccharide cellobiose (Howell and Stuck, 1975). In addition to cellobiose, hydrolysis of biomass yields varying amounts of D-glucose, D-xylose, D-mannose, D-galactose and L-arabinose, depending upon the hydrolysis procedure and the nature of the feedstock (Bisaria and Ghose, 1981). The extent to which these sugars can be fermented to ethanol by yeasts is therefore of paramount importance for commercial exploitation of lignocellulosic biomass to fuel energy.

A few cellobiose-fermenting yeasts have been identified, namely the Torulopsis, Brettanomyces and Candida species (Freer and Detroy, 1983, Kilian et al., 1983, Lastick et al., 1983). Other investigators have shown that yeasts which can ferment cellobiose to ethanol have a poor

capacity for xylose fermentation (Maleszka et al., 1982). Recently, strains of Candida tenuis and Pichia stipitis have been identified that ferment cellobiose as well as xylose (Toivola et al., 1984, Du Preez et al., 1986). The objective of the present study is to identify pentose-fermenting yeasts which can convert cellobiose as well as other wood sugars simultaneously and efficiently to ethanol. Such yeasts can find direct application in the simultaneous saccharification and fermentation (SSF) conversion process of cellulosics to ethanol.

MATERIALS AND METHODS

Microorganisms: Candida shehatae CSIR-117, Candida shehatae CSIR-57 and Pichia stipitis CSIR-Y633 were supplied by Prof. J.P. van der Walt, South African Council for Scientific and Industrial Research (CSIR), Pretoria. P. stipitis CBS 5776 and P. stipitis CBS 5773 were obtained from Central Bureau voor Schimmelcultures (CBS), Yeast Division, Delft, The Netherlands. The strain of Candida shehatae was ATCC 22984 from the American Type Culture Collecton Center, Bethesda. All yeasts were maintained on YMP agar slants with xylose as the carbon source.
Culture media: Du Preez's Medium CA, containing casamino acids (Difco, vitamin free), added vitamins and minerals (Du Preez et al., 1986) and adjusted to pH 5.0, was used. Cellobiose was used at 25 g/L. The wood sugar mixtures used as substrates contained D-glucose, D-xylose, D-cellobiose, D-galactose and D-mannose. The sugars were autoclaved separately from the rest of the medium constituents.
Inoculum and fermentation conditions: The inoculum was prepared in 250 mL Erlenmeyer flasks with 100 mL of medium containing 20 g/L D-xylose as the carbon source and grown for 72 h at 30°C on a rotary shaker at 150 rpm. Cells were harvested by centrifugation, washed twice with saline, and inoculated in fermentation flasks at a dry cell concentration of 0.25 g/L. All shake flask fermentations were performed under semiaerobic conditions at 30°C as described earlier (Wayman and Parekh, 1985).
Analysis: Ethanol was determined using gas chromatography and individual wood sugars using High Performance Liquid Chromatography (Waters) fitted with a Bio-Rad Aminex HPX-87P column, and using water at 65°C as the mobile phase. Washed yeast cell dry weight was determined by a standard curve relating biomass to absorbance at 660 nm.

RESULTS AND DISCUSSION

Cellobiose fermentation: Results of the cellobiose fermentations are summarized in Table 1. C. shehatae Y117 and C. shehatae 57 utilized the disaccharide for growth but failed to produce ethanol. The results are in agreement with Du Preez et al., (1986), except that C. shehatae ATCC 22984 produced more ethanol than expected, suggesting that more D-cellobiose fermenting strains exist than have been identified to date. All the Pichia stipitis strains tested produced ethanol from cellobiose. The highest efficiency of fermentation, 0.41 g ethanol/g cellobiose, was obtained with P. stipitis CBS 5776, which fermented all of the cellobiose within 48 hrs. The yield of ethanol is higher than that reported earlier

Table 1

Ethanol Production from 25 g/L cellobiose by various yeasts.

Yeast strains	Fermentation time h	Substrate utilized %	Final ethanol concentration g/L	Ethanol yield (g ethanol/ g substrate utilized)
C. shehatae Y117	64	22	0	0
C. shehatae 57	64	15	0	0
C. shehatae ATCC 22984	48	100	5.8	0.23
P. stipitis CBS 5776	48	100	10.3	0.41
P. stipitis CBS 5773	64	92	2	0.09
P. stipitis Y633	48	100	7.4	0.30

Table 2

Ethanol production by various yeasts from 60 g/L wood sugar mixture (D-xylose 15 g/L, D-glucose 15 g/L, D-mannose 10 g/L, D-galactose 10 g/L and D-cellobiose 10 g/L)

Yeast strains	Fermentation time h	Substrate utilized %	Cellobiose utilized %	Ethanol g/L	Ethanol yield (g ethanol/ g substrate consumed)
C. shehatae Y117	64	84.3	6.0	18.3	0.36
C. shehatae 57	64	84.0	5.0	18.0	0.36
C. shehatae ATCC 22984	64	87.5	25.0	19.8	0.38
P. stipitis CBS 5776	48	100	100	26.0	0.43
P. stipitis CBS 5773	64	86.3	18.0	18.5	0.36
P. stipitis Y633	64	95	70.0	22.2	0.39

for P. stipitis Y633 (Du Preez et al., 1986), and compares favourably with C. wikerhamii, one of the more efficient cellobiose fermenting yeasts (Freer and Detroy, 1983) with a rapid rate of ethanol production and an ethanol yield coefficient of 0.41 (Kilian et al., 1983). C. wikerhamii produced ethanol from cellobiose only under anaerobic conditions (Kilian et al., 1983) and has not yet been reported to ferment pentose sugars such as those in wood hydrolysates.

Fermentation of wood sugar mixtures: Five wood sugar mixtures were used in order to determine the yields that could be expected during fermentation of wood hydrolysates. The results (Table 2) show that all of these yeasts fermented 84% or more of these sugars, including the xylose, but that C. shehatae Y117 and C. shehatae 57 utilized very little of the cellobiose. P. stipitis CBS 5776 fermented these sugars completely in 48 hrs with a yield of 0.43 g ethanol/g sugar consumed representing about 86% of theory. P. stipitis Y633 was slower and utilized only 70% of the cellobiose in 64 h, at a yield of 0.39. The two other yeasts, P. stipitis CBS 5773 and C. shehatae ATCC 22984, were also less efficient in fermentation of cellobiose than P. stipitis CBS 5776, although they were able to convert the other sugars to ethanol.

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REFERENCES

Bisaria, V.S. and , Ghose, T.K. (1981). Enzyme Microb. Technol. 3:90-104.

Du Preez, J.C., Bosch, M. and Prior, B.A. (1986). J. Appl. Microbiol. Biotechnol. 23:228-233.

Freer, S.N. and Detroy, R.W. (1983). Biotechnol. Bioeng. 25:541-557.

Goldstein, I.S. (1981). "Organic Chemicals from Biomass", ed. I.S. Goldstein (CRC Press, Boca Raton, FL., U.S.A.) p. 101.

Howell, J.A. and Stuck, J.D. (1975). Biotechnol. Bioeng. 17:873-893.

Kilian, S.G., Prior, B.A., Potgieter, H.J., Du Preez, J.C. (1983). J. Appl. Microbiol. Biotechnol. 17:281-286.

Lastick, S.M., Spindler, D.D. and Grohman, K. (1983). "Wood and Agricultural Residues, research on use for feed, fuels and chemicals", ed. E.J. Soltes, Academic Press, N.Y., London, pp. 239-251.

Maleszka, R., Wang, P.K. and Schneider, H. (1982). Biotechn. Letts. 4:133-136.

Toivola, A., Yarrow, W., Van der Bosch, E., Van Dijken, J.P., and Scheffers, W.A. (1984). Appl. Environ. Microbiol. 47:1221-1223.

Wayman, M. and Parekh, S. (1985). Biotechn. Letts. 7:909-912.

Wayman, M., Tallevi, A. and Winsborrow, B.C. (1984). Biomass. 6:183-191.

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SO_2-CATALYSED PREHYDROLYSIS OF CONIFEROUS WOOD FOR ETHANOL PRODUCTION

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SUMMARY: This paper reports studies of large scale, 1500 kg/h, SO_2-catalysed prehydrolysis of coniferous wood chips, samples then being hydrolyzed by a wood-saccharifying enzyme system followed by fermentation to ethanol in the laboratory. Hemicellulose hydrolysis using SO_2 catalyst (prehydrolysis) was found to be more effective than steam alone (autohydrolysis). Prehydrolysis time was 2 min, with steam pressure at 1.2 to 1.7 MPa (175 to 250 psig), and SO_2 catalyst 2.0 to 2.6% on dry wood. The amount of sugars recovered upon enzyme saccharification of the prehydrolysed wood was about 70% of the weight of the wood. When these combined hemicellulose and cellulose sugars were fermented by a pentose-fermenting strain of yeast, Pichia stipitis R, 372 L ethanol/tonne of (dry) wood was obtained.

INTRODUCTION: Lignocellulosic biomass such as forest and agricultural residues are used or proposed as raw materials for chemicals and fuels production. Before they can be hydrolysed to fermentable sugars, these feedstocks require pretreatment to render them susceptible to attack by hydrolytic agents. During pretreatment, hemicellulose is hydrolysed, either by steam (autohydrolysis), or by a catalyst (prehydrolysis). The fibrous residue may then be hydrolysed by acid or enzymes (Wayman et al. 1979). Autohydrolysis is effective in hydrolysing hemicelluloses of deciduous woods, such as aspen, and of agricultural crop residues, but has not yet been found useful for coniferous woods such as pine, which are the main residues of forest industries. The merits of using SO_2 as catalyst for prehydrolysis of coniferous wood were explored and reported (Wayman et al. 1984) with encouraging results. The present paper reports conditions for SO_2 prehydrolysis of coniferous wood chips on a large scale, direct enzyme saccharification of the cellulosic residues so produced and fermentation of the combined hydrolysates to ethanol by an efficient pentose-fermenting yeast, Pichia stipitis R (Parekh et al. 1986), resulting in high yields of ethanol.

MATERIALS AND METHODS: Wood analysis: Mixed pine and spruce chips from the Eastern Townships of Quebec were used with the following composition, in % of dry weight: polysaccharides, 70, lignin 27, extractives 3. Of the polysaccharides, 48% was glucan, 22% non-glucan.

Prehydrolysis: Commercial chips, approximately 19 x 13 x 3 mm, were fed to a Stake II reactor installed at the Université de Sherbrooke. This was set to operate at the rate of 1500 kg/h to give 2 min residence time. Direct steam and SO_2 were added, steam pressure being controlled from 1.21 to 1.72 MPa (175 to 250 psig), and SO_2 addition being 2.0 to 2.6% on (dry) wood. In one run, no SO_2 was added. The so-treated chips fragment on release of pressure on discharge. The residues so obtained were not washed to remove hemicellulose, as is often done, but saccharified directly.

Enzyme saccharification: Enzymatic hydrolysis was carried out on samples of prehydrolysed wood in 500 mL flasks at 50°C in a water bath, agitated at 170 rpm. The pH was adjusted to 4.8 with lime, the liquid/solid ratio being 5.2/1, that is 16% dry solids. The wood-saccharifying enzyme system used contained cellulase (Meiji Seika, 0.02 g/g wood), hemicellulase (Miles, 0.01 g/g wood) and cellobiase (Novo, 0.01 g/g wood), as well as Vitamin B_{12} and trace elements as promoters. Since the rate of enzyme hydrolysis decreases markedly with time, half the enzyme was added initially, and the other half, being fresh enzyme, at 24 hours, a procedure (Wayman et al. 1979) which resulted in excellent yields of sugars.

Microorganism: A pentose-fermenting strain of Pichia stipitis CBS 5776 was acclimatized to wood hydrolysates by semiaerobic cultivation for 24 h, centrifuging and recycling the yeast repeatedly. The so-called R strain so obtained was maintained on YMP agar with 2% xylose as carbon source.

Fermentation: Inoculum was prepared in YMP medium containing 70:30 glucose: xylose, in a 2 L flask on a rotary shaker at 30°C. Cells were harvested by centrifugation, washed twice with saline and inoculated into the fermentation flasks to give cell concentrations of 16 g/L. All fermentations were performed under semiaerobic conditions (Wayman and Parekh, 1985) at 30°C for 70 h.

Analysis: Ethanol was determined by GC, and the individual wood sugars by HPLC (Waters Sugar Analyser, Bio-Rad Aminex HPX-87P column using water at 65°C as the mobile phase).

RESULTS: Table 1 presents the results of 4 prehydrolysis runs. In the presence of SO_2 as catalyst, steam at 1.72 MPa (250 psig) and 2.0% SO_2 on wood, or steam at 1.38 MPa (200 psig) and 2.6% SO_2 on wood, resulted in excellent hydrolysis of hemicellulose, about 89% of theory, and nearly all of the hemisugars appeared as monomers. On decreasing steam pressure, and therefore temperature, while maintaining 2 min processing time, the yields of hemisugars obtained at 1.21 MPa (175 psig) decreased to about 75% of theory. Autohydrolysis with steam at 1.72 MPa (250 psig) was less effective than SO_2 prehydrolysis, only 77% of sugars being recovered as soluble carbohydrates, and more than 30% of these were oligomers.

Table 2 presents the yields of wood sugars obtained after enzymatic saccharification of the cellulosic residue of prehydrolysis. The use of

TABLE 1. Analysis of prehydrolysates of coniferous wood

Run No.	Prehydrolysis Conditions	Glucose	Total Sugars	Residue
	all 2 minutes	percent on (dry) wood		
1	2.0% SO_2 on wood, steam at 1.72 MPa (250 psig)			
	Monomer	5.41	26.56	71.3
	Oligomer	0.14	0.54	
	Total	5.55	27.10	
	% of theory: glucose 10.5; non-glucose sugars 89.0			
2	2.6% SO_2 on wood, steam at 1.38 MPa (200 psig)			
	Monomer	5.10	21.94	72.9
	Oligomer	0.10	4.82	
	Total	5.20	26.76	
	% of theory: glucose 9.8; non-glucose sugars 89.1			
3	2.6% on wood steam at 1.21 MPa (175 psig)			
	Monomer	4.15	20.63	74.0
	Oligomer	0.29	2.08	
	Total	4.44	22.71	
	% of theory: glucose 8.4; non-glucose sugars 75.5			
4	no SO_2 steam at 1.72 MPa (250 psig)			
	Monomer	3.24	15.79	75.6
	Oligomer	0.93	6.94	
	Total	4.17	22.73	
	% of theory: glucose 7.8; non-glucose sugars 76.7.			

TABLE 2. Fermentation of combined hemicellulose and cellulose hydrolysates of coniferous wood by *Pichia stipitis* R

	Total Sugars				ethanol yield	
		before fermentation	after fermentation	ethanol	g ethanol/ g sugars	L/tonne
Run No.	% on wood	g/L	g/L	g/L	consumed	of wood
1	71.7	134	6	55	0.43	372
2	68.7	126	6	52	0.43	352
3	61.5	117	7	49	0.45	326
4	52.7	109	9	44	0.44	273

2.0% SO_2 with steam at 1.72 MPa (250 psig) or 2.6% SO_2 at 1.38 MPa (200 psig) resulted in about 70% of the wood being recovered as sugars. The maximum sugar recovery which can be obtained from this wood based on its chemical composition is 77%, so these results are somewhat over 90% of theory, which is probably the highest yet reported from coniferous wood. Lowering the steam pressure while maintaining processing time at 2 min and SO_2 at 2.6% on wood resulted in sugar recovery of only 61% of the wood. The use of steam alone resulted in recovery of only 53% of the wood as sugars.

DISCUSSION: On fermentation by Pichia stipitis R, more than 93% of the sugars were utilized, with ethanol yields all in a narrow range, 0.43 to 0.45 g ethanol/g sugar consumed, this being about 90% of the theoretical efficiency of ethanol formation from these sugars. When the wood was prehydrolysed with 2.0% SO_2 and steam at 1.72 MPa (250 psig), ethanol concentration was 55 g/L, which is 7.0% by volume, a good value for economic distillation. The yield of ethanol was 372 L/tonne of wood. The theoretical maximum obtainable based on wood composition calculates to be 478 L/tonne, so these results are about 75% of theory, which can be considered quite satisfactory. The autohydrolysis run produced only 273 L ethanol/tonne of wood, or about 57% of theory. These results demonstrate that a commercial scale steam pretreatment system using a simple gaseous catalyst at low concentration can produce an excellent substrate for subsequent enzymatic hydrolysis and fermentation to ethanol using coniferous species as feedstock. A system of commercial enzymes - cellulase, hemicellulase and cellobiase - worked successfully on the SO_2 pretreated material, without water extraction with no evidence of toxicity or inhibition of either the enzymes or the P. stipitis R which together gave a yield of better than 70% of that theoretically possible on the basis of the sugar content of the original feedstock. The efficient utilisation of the wood sugars by the P. stipitis R at realistic ethanol concentrations and the elimination of a wash step for the SO_2 pretreated material has major implications for the development of a viable technology to produce ethanol from coniferous wastes.

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REFERENCES

Parekh, S., Yu, S. and Wayman, M. 1986. J. Appl. MIcrobiol. and Biotechn. in press.

Wayman, M., Lora, J.H. and Gulbinas, E. 1979. Chemistry for Energy, ACS Symposium Series Monograph No. 90, 183-201.

Wayman, M. and Parekh, S. 1985. Biotechn. Letts. 7:909-912.

Wayman, M., Tallevi, A. and Winsborrow, B. 1984. Biomass 6:183-191.

10.12

Isoenzyme and Protein Patterns of Pentose-fermenting Yeasts After Adaptation to Wood Hydrolysates

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Summary

Soluble proteins were extracted from the vegetative cells of two pentose-fermenting yeasts, Candida shehatae and Pichia stipitis, and their adapted strains, C. shehatae R and P. stipitis R respectively. Isoenzyme patterns, protein patterns and two-dimensional polypeptide mapping of these four strains were compared by polyacrylamide gel electrophoresis. The two adapted strains showed great similarity in two-dimensional polypeptide mapping, the pattern of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), isoelectrofocusing (IEF), and isoenzymes. The two starting strains of these yeasts had their own characteristic electrophoretic patterns. The adapted strains showed the presence of 3 alcohol dehydrogenase isoenzymes compared to 1 for the starting strains, as well as much higher activity of malate dehydrogenase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which further formation of pyruvate and ethanol.

Introduction

Agricutural and forest biomass have a considerable content of hemicellulose components, and the economy of ethanol production from biomass would be much improved by efficient fermentation of the pentoses and other carbohydrates. In our studies of liquid fuel production from biomass, especially from wood hydrolysates and spent sulphite liquor, two pentose-fermenting yeasts, Candida shehatae Buckley et van Uden and Pichia stipitis Pignal were used (Wayman and Parekh 1985; Wayman and Tsuyuki 1986; Yu and Wayman 1986). However, the fermentations were slow and incomplete, due to high levels of fermentation inhibitors and catabolic repression of xylose-utilization resulting from the presence of easily assimilated hexose sugars. Of great importance in confering resistance to inhibitors and improving the rate of fermentation was our practice of recycling the yeasts from each fermentation as the inocula for the next fermentation (Lee et al. 1986). The recycling was carried out repeatedly under "semiaerobic" conditions, which are a compromise, maximum ethanol production and pentose-utilization being achieved by the supply of air at a strictly controlled rate. These recycled yeasts are able to tolerate high levels of inhibitors and to ferment both hexoses and pentoses efficiently and rapidly, resulting in greatly improved substrate utilization and ethanol production compared to the starting strains. For example, the yeasts recycled in aspen hydrolysate improved the fermentation efficiency, compared to the

starting strains, from 0.39-0.41 to 0.45-0.47 g ethanol/g sugar consumed, with sugar utilization being increased from 71-78% to 93-95% (Parekh et al. 1986; Yu et al. 1986). In addition, significant morphological differences between the adapted strains and the starting strains were seen (Wayman and Yu, unpublished). These findings encouraged us to investigate further the differences in other characteristics of these yeast strains.

Gel electrophoretic patterns of soluble proteins extracted from fungal mycelium have received considerable attention and have shown some success in differentiating species, formae species, or physiological races of various fungi (Chang et al. 1962; Clare and Zentmeyer 1966; Durbin 1966; Macko et al. 1967; Clare et al. 1968; Gill and Powell 1968a; Hall 1969; Stipes 1970; Milton et al. 1971).

The objective of the present study was to investigate whether isoenzyme and protein patterns are of value for the characterization of the adapted and starting yeast strains of C. shehatae and P. stipitis, and for interpretation of their different behaviour in fermentation.

Materials and Methods

Microorganism and culture conditions

The starting strains of C. shehatae (ATCC 22984) and P. stipitis (CBS 5776) were originally obtained from American Type Culture Collection and Centraal Bureau von Schimmelcultures,

respectively. These cultures were plated out and colonies derived from single cells were plated out again to ensure purity of the culture. Great care was taken to avoid contamination. The adapted strains were obtained according to the methods developed by Parekh et al. (1986) and Yu et al. (1986) and designated here as C. shehatae R and P. stipitis R for C. shehatae (ATCC 22984) and P. stipitis (CBS 5776) respectively. All of them were grown in sugar medium containing 2% glucose, 1% xylose, 0.3% yeast extract, 0.3% malt extract and 0.5% peptone, at a starting pH of 5.5. Incubation was performed in 250 mL shake flasks containing 100 mL medium, with porous foam closures, at 150 rpm and 30°C for 3 days. At the end of incubation, yeast cells were harvested by centrifugation with a Sorvall RC-5B refrigerated centrifuge at 640 xG for 10 min.

Protein extraction

The extraction procedure for soluble proteins was similar to that used by Stipes (1970). Yeast cells harvested by centrifugation were ground for 1 min at top speed with a Polytron homogenizer (Brinkmann Instruments) in a dry ice-acetone mixture (15 mL/g wet weight of cells). The resulting slurry was filtered on Whatman No. 1 filter paper, and washed with 100 mL of dry ice-acetone mixture. This dry fungal powder is referred to as acetone powder. Seventy-five mg of acetone powder was extracted for soluble proteins in 1 mL of a 4 mM $NaHCO_3$ solution (pH 7.6) at 4°C. The extract was centrifuged at 40,000 xG for 1 hour. The

resultant pellet was discarded. The quantity of soluble proteins in the supernatant was determined by Bradford's method (1976) using bovine crystalized albumin as standard and Coomassie blue R-250 as dye reagent (Bio-Rad protein assay kit, Bio-Rad Laboratories).

Electrophoresis

Electrophoretic techniques used in this study were performed based on the protocol described by Laemmli (1970).

For the detection of isoenzymes, electrophoresis was carried out with a Bio-Rad Protean Dual 16 cm slab cell. The gels contained 7.5% acrylamide, 0.375 M Tris-HCl (pH 8.9) and were chemically polymerized by the addition of 0.005% ammonium persulfate (AP) (w/v) and 0.005% N,N,N,N-tetramethylethylenediamine (TEMED). Stacking gels of 5% acrylamide were laid on the top of the separation gels. Samples for electrophoresis were prepared by adding equal amounts of sample buffer containing 20% glycerol, 0.62 M Tris-HCl (pH 6.8), and bromophenol blue as tracking dye to the protein extracts. Sample solutions standardized to have 100 µg of protein per sample were loaded on the gel. The electrode buffer solution consisted of 0.052 M Tris-glycine (pH 8.9).

For the detection of soluble proteins, electrophoresis was carried out in sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gradient slab gel. Slab gels, 23 x 14 x 0.5 cm, were made with a Pharmacia mixer GM-1 equipped with a peristalitic pump P-1, at a flow rate of 5 mL per min. The concentration of the gradient gel

ranged from 8 to 15%. A 4 cm long stacking gel (5%) was laid on the top of the running gel. The running gel contained 0.375 M Tris-HCl (pH 8.9) and 0.1% SDS. The gel was polymerized chemically by adding 0.05% AP and 0.05% TEMED. The electrode buffer consisted of 0.052 M Tris-glycine (pH 8.9) and 0.1% SDS. Samples for electrophoresis were prepared by adding equal amounts of sample buffer containing 20% glycerol, 2% SDS, 2% 2-mercaptoethanol, 0.062 M Tris-HCl (pH 6.8) and bromophenol blue to the protein extract. Immediately before loading on the gel, the sample solutions were boiled for 1 min. Sample solutions standardized to have 100 µg protein per sample were loaded on the gel. Bio-Rad SDS-PAGE low molecular weight markers were used as references.

For isoelectrofocusing (IEF), 10 mg of lyophilized proteins were suspended in 1 mL of sample solution containing 0.4% Bio-Lyte (pH 3-10) and 1.6% Bio-Lyte (pH 4-6) (Bio-Rad Laboratories) and centrifuged to remove particulates. Isoelectric focusing gel contained 4.85% acrylamide, 0.15% N,N-methylene-bis-acrylamide (Bis), 0.4% Bio-Lyte (pH 3-10), 1.6% Bio-Lyte (pH 4-6), 0.0005% riboflavin-5-phosphate and 5% glycerol. Slab gels were polymerized under fluorescent light. All chemicals were dissolved in deionized water. Ten microliters of each protein sample were absorbed onto pieces of filter paper (6 x 6 mm square) which was laid onto thin gel (0.8 mm in thickness) near the cathode. The inter-electrode distance was 100 mm. Filter strips soaked in 1 N H_3PO_4 and 1 N NaOH were used as anodic and cathodic solution respectively.

Isoelectrofocusing was carried out with a Bio-Rad horizontal electrophoresis cell (Model 1415) at a constant power of 5 watts for 3-6 hours. Determination of the pH gradient was made, after completion of electrophoresis, by cutting a gel strip into 1 cm segments, equilibrating the segments with deionized water, and measuring the pH. After focusing, the gel was first fixed with a fixative (4% sulfosalicylic acid, 12.5% trichloracetic acid and 30% methanol) for 1 hour and then stained overnight in a solution containing 27% ethanol, 10% acetic acid, 0.04% Coomassie brilliant blue R-250 and 0.5% $CuSO_4$. The gel was destained in a solution of 12% ethanol, 7% acetic acid and 0.5% $CuSO_4$ until the background was clear. The gel can then be stored indefinitely in a solution of 12% ethanol and 7% acetic acid.

Two-dimensional electrophoresis of proteins was performed using a modification of the method of O'Farrell (1975). The proteins were first separated in glass tubes (140 mm long; i.d. 5 mm) filled to 110 mm with a gel solution as described above (see IEF section). After polymerization, sample solutions containing 450 µg proteins were laid directly on the gel surface. Isoelectrofocusing was carried out in a Pharmacia GE-2/4 gel apparatus with 0.06% phosphoric acid as the lower (anode) electrolyte and 0.1 N NaOH as the upper (cathode) electrolyte at 15°C for 12-14 hours at 100 volts followed by 1 hour at 200 volts. After electrofocusing, gels were equilibrated for 2 hours in a solution containing 0.063 M Tris-HCl (pH 6.8), 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol and

0.01% bromophenol blue. Buffer equilibrated gels were then laid lengthwise on the top of the SDS-PAGE gradient gels and sealed in placed by 1% agarose. The composition and range of gradient in the gels were the same as described (see SDS-PAGE section). Gels were run at a constant 200 volts in a Bio-Rad Protean Dual 16 cm slab cell.

Staining for isoenzymes and SDS-PAGE proteins

After electrophoresis the gels were removed from the glass plates and placed directly into substrate solutions for isoenzyme staining. Isoenzymes were visualized by the methods described by Shaw and Prasad (1970), Siciliano and Shaw (1976), Mayer and Harel (1979), Kieliszewska-Rokicka (1980) and Hammerschmidt et al. (1982). Substrates used for enzyme reactions were obtained from Sigma.

For SDS-PAGE protein patterns, the gels were first stained with 0.01% Coomassie brilliant blue R-250 in PAGE fixative (methanol:acetic acid:water = 45:10:45) at 60°C for 1 hour and then fixed in PAGE fixative at 60°C for another hour. The gels were destained in a solution of 5% methanol and 7% acetic acid until the background was clear.

Results

Detection of izoenzymes on gels

The isoenzymes which were electrophoretically analysed in this study were alcohol dehydrogenase (ADH), aldolase (ALD), esterase (EST), glucose-6-phosphate dehydrogenase (G6PDH), hexokinase (HK), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), peroxidase (PO), 6-phosphogluconate dehydrogenase (6PGDH), phosphoglucomutase (PGM), succinate dehydrogenase (SDH) and tetrazolium oxidase (TO). These isoenzymes have been used routinely in our laboratory to differentiate fungal strains.

Figure 1 shows the isoenzyme patterns of these 12 enzymes. The starting strains of these two yeasts had their own characteristic banding patterns whereas the isoenzyme patterns of the adapted strains exhibited a remarkable similarity.

Alcohol dehydrogenase

The starting strains of C. shehatae and P. stipitis each had one band of alcohol dehydrogenase with different mobility on the gel while the adapted strains each exhibited 3 identical isoenzyme bands (Figure 1a).

Aldolase

A total of 4 distinct enzyme bands were detected for both recycled strains (Figure 1b). They were similar in the mobility as well as the density of reaction. The two starting strains could be distinguished by their own banding patterns.

Esterase

Two adapted strains showed a similar banding pattern of esterase. One dark and two light reaction bands could be visualized for these two strains (Figure 1c). The two starting strains showed rather weak and different bands as compared with the adapted ones.

Glucose-6-phosphate dehydrogenase

While both adapted strains had a similar enzyme reaction zone of glucose-6-phosphate dehydrogenase, the starting strains of C. shehatae and P. stipitis showed different bands of enzyme reaction (Figure 1d).

Hexokinase

Both adapted strains showed at least 3 enzyme bands with the same mobility on the gel (Figure 1e). The two starting strains exhibited their own characteristic enzyme patterns which were different from the adapted ones.

Isocitrate dehydrogenase

A single band of isocitrate dehydrogenase was detected for both starting and adapted strains (Figure 1f). The banding pattern of this enzyme in the two starting strains not only differed from each other but also from the adapted ones.

Malate dehydrogenase

Both starting and adapted strains showed a similar banding pattern of malate dehydrogenase (Figure 1g). The adapted strain of C. shehatae and P. stipitis exhibited very much stronger enzyme activity than the starting strains. C. shehatae had little activity for this enzyme.

Peroxidase

One band of peroxidase activity was detected for each strain (Figure 1h). The adapted strains exhibited a similar banding pattern while the two starting strains showed their own characteristic enzyme activity.

Phosphoglucomutase

The starting strains of C. shehatae and P. stipitis each showed one enzyme band on different positions of the gel (Figure 1i). Little or no enzyme reaction could be detected for the adapted strains.

6-phosphogluconate dehydrogenase

Only one band of 6-phosphogluconate dehydrogenase with low enzyme activity was detected in the starting strain of C. shehatae. The adapted strains of this yeast exhibited a very high enzyme reaction zone near the top portion of the gel (Figure 1j). A similar reaction zone was also detected for the adapted strain of

P. stipitis. The starting strain of P. stipitis showed 3 bands of enzyme activities different from other 3 strains.

Succinate dehydrogenase

Only one band of enzyme activity was detected for all strains. However, two adapted strains showed higher enzyme reaction than the two starting strains (Figure 1k).

Tetrazolium oxidase

The activity of tetrazolium oxidase is expressed as a white area on the light blue background on the gel. While the two starting strains depicted their own banding patterns, the adapted strains showed the same reaction zone (Figure 1l).

Soluble patterns on isoelectrofocusing gel

Isoelectrofocusing fractionated the proteins into narrow, concentrated bands at their isoelectric points. As can be seen in Figure 3, about 30 to 40 Coomassie blue staining protein bands were discernible for each strain between pH 3.7 to 6.2. C. shehatae R and P. stipitis R possessed so many similar bands at different pH values that it is difficult to differentiate them from each other, whereas their starting strains each had their characteristic electrofocusing pattern.

Two-dimensional electrophoresis of soluble proteins

Polypeptide mapping of the 4 strains is shown in Figure 4 and Figure 5. C. shehatae ATCC 22984 differed from P. stipitis CBS 5776 in polypeptide mapping by having many more protein spots above 40 K daltons molecular weight, particularly between pH 4.5 to 5.5. In addition, C. shehatae ATCC 22984 could be recognized by the presence of such protein spots as 1, 2, 3, 4, 5 and 6 (Figure 4A). P. stipitis CBS 5776 possessed other proteins such as a, b, c, d, and e (Figure 4B). Figure 5 showed that the two recycled strains, C. shehatae R and P. stipitis R, possessed very similar soluble proteins. They were characterized by the presence of at least 24 apparently identical protein spots. This figure also showed that many proteins present in the starting strains were suppressed after they were recycled in wood hydrolysates, particularly in the region above 31 K dalton molecular weight. Although C. shehatae R and P. stipitis R showed great similarities in SDS-PAGE, IEF and isoenzyme patterns, they could be distinguished from each other by their specific protein spots (Figure 5, arrows).

Discussion

The results of this study show that the adapted strains, C. shehatae R and P. stipitis R, are quite different from their starting strains. This agrees with the results obtained from fermentation (Parekh et al. 1986) in which the adapted strains fermented xylose and wood sugars more rapidly and more efficiently,

and were able to tolerate higher concentrations of fermentation inhibitors than their starting strains.

The suggestion has been made (Kreger Van Rij 1971) that C. shehatae is an imperfect form of P. stipitis, differing only in loss of sporogenic ability. The results of the present study show that the two culture collection species differ markedly in isoenzyme patterns, and in protein patterns as shown by one- and two-dimensional electrophoresis and by isoelectrofocusing. These differences are shown in each of the 5 figures. On the basis of these considerable differences in isoenzyme and protein constitutions, we must consider the two culture collection species as distinctly different.

The adapted strains, while having remarkable similarities, are also different from each other, especially in the protein patterns shown by two-dimensional electrophoresis. The specific polypeptides common to both recycled strains may represent gene products that confer increased fitness in the fermentation broth. On the other hand, C. shehatae R may be expected to differ from P. stipitis R for gene products that were not selected for in the broth, and we have observed that such is indeed the case (Figure 5). The morphology, physiology and genetics of these 4 strains are being studied. Preliminary indications are that the recycled strains are identical with each other, and are different from the starting strains in these characteristics. As yet the recycled

strains have not been identified as members of any described yeast species. This work will be reported in due course.

The purpose of recycling C. shehatae and P. stipitis in spent sulphite liquor and wood hydrolysates was to enhance resistance to toxic substrates and to improve the rate of sugar utilization. There are many chemical substances, such as lignin, phenolic compounds, acetic acid, formic acid, and furfural present in spent sulphite liquor and wood hydrolysates, which are generally considered toxic to microorganisms. Such a chemical environment probably exerted a strong selection pressure on the yeasts; and the enzymes necessary for best cell growth and substrate utilization in the environnment were therefore induced. This assumption is suported by the similarities in polypeptide mapping of C. shehatae R and P. stipitis R and the presence of some characteristic proteins, for instance, protein bands A, C, G and H, which are located in the molecular weight region of most enzymes. In addition, a striking similarity in banding patterns and enzyme reactions of those enzymes tested in glycolytic, citric acid and pentose phosphate pathways further supported this hypothesis.

One of the most striking features of C. shehatae and P. stipitis and their recycled strains is the banding pattern of alcohol dehydrogenase. While the parent strains had only one isoenzyme band, the recycled strains showed the presence of 3 distinct enzyme reactions of alcohol dehydrogenase (Figure 1a). Final decarboxylation of pyruvate to the formation of ethanol is

mediated by this enzyme. The activity of alcohol dehydrogenase together with the production of other new isoenzymes in glycolytic, citric acid and pentose phosphate pathways of the recycled yeasts may contribute to the greater ethanol production and efficient utilization of carbohydrates present in wood hydrolysates and spent sulphite liquor.

Also remarkable is the very high activity of IDH and MDH in the adapted strains, in contrast with the low levels in the starting strains, especially in C. shehatae ATCC 22984. Isocitrate and malate are part of the respiratory pool, the TCA cycle, and the effect of the high concentrations of these enzymes may be to further conversion of isocitrate and malate to pyruvate, which is the precursor of ethanol.

It is suggested that the superior fermentation efficiency of C. shehatae R and P. stipitis R is due to the presence of characteristic proteins such as those in bands A, C, G and H of Figure 2a, which are in the molecular weight range of most enzymes. Some of these enzymes may be absent in the culture collection strains, or present but repressed, while the recycling process derepresses the enzymes necessary for efficient fermentation of xylose.

The metabolism of xylose by yeasts proceeds via xylose reductase and xylitol dehydrogenase to xylulose (Gong and Tsao 1983). Xylose reductase is NADPH-dependent, the required NADPH being supplied by the pentose phosphate pathway via GPDH and PGDH

(Bruinenberg et al. 1983). Both adapted strains had high GPDH and PGDH ativity, while the starting strains showed much lower activity of these two enzymes, especially GPDH of C. shehatae ATCC 22984 and the PGDH of P. stipitis CBS 5776. While the starting strains are capable of fermenting xylose, the recycled strains, with their better supply of the necessary enzymes, do so with significantly higher efficiency.

BRADFORD, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

BRUINENBERG, P.M., J.. VAN DIJKEN and W.A. SHEFFERS. 1983. A theoretical analysis of NADPH production and consumption in yeasts. J. Gen. Microbiol. 129: 953-964.

CHANG, L.O., A.M. SRB and F.C. STEWARD. Electrophoretic separations of the soluble proteins of Neurospora. Nature 193: 756-759.

CLARE, B.G. and G.A. ZENTMEYER. 1966. Starch gel electrophoresis of proteins from species of Phytophthora. Phytopathology, 56: 1334-1335.

CLARE, B.G., N.T. FLENTJE and M.R. ATKINSON. 1968. Electrophoretic patterns of oxidoreductase and other proteins as criteria in fungal taxonomy. Aust. J. Biol. Sci. 21: 275-295.

DURBIN, R.D. 1966. Comparative gel electrophoretic investigations of protein patterns of septoria species. Nature 210: 1186-1187.

GILLS, H.S. and D. POWELL. 1968a. Polyacrylamide gel electrophoresis of physiologic races A-1 to A-8 of Phytophthora fragariae. Phytopathology 58: 722-723.

-----. 1968b. The use of polyacrylamide disc electrophoresis in delimiting three species of Phytophthora. Phytopathol. Z. 63: 23-29.

GONG, C.S. and G.T. TSAO. 1983. Conversion of D-xylose to ethanol by yests. In Proceedings Intern. Symp. on Ethanol from Biomass. Ed. H.E. Duckworth and E.A. Thompson. Royal Soc. Canada, Ottawa, pp. 525-555.

HALL, R. 1969. Verticillium albo-atrum and V. dahliae distinguished by acrylamide gel electrophoresis of proteins. Can. J. Bot. 47: 2110-2111.

HAMMERSCHMIDT, R., E.M. NUCKLES and J. KUC. 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to Collectotrichum lagenarium. Physiol. Plant Pathol. 20: 73-82.

KIELISZEWSKA-ROKICKA, B. 1980. Isoenzymes of peroxidase, indole-3-acetic acid oxidase and polyphenoloxidase of poplar and pine. Acta Physil. Plant. 2: 195-207.

KREGER-VAN RIJ, N.J.W. 1971. Pichia Hansen. In The Yeasts-a toxonomic study, second edition. Ed. by J. Lodder, North-Holland Publishing Comp., Amsterdam, pp. 533-535.

LAEMMLI, V.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophase T4. Nature 227: 681-685.

LEE, H., P. BIELY, R.K. LATTA, M.F.S. BARBOSA and H. SCHNEIDER. 1986. Utilization of xylan by yeasts and its conversion to ethanol by Pichia stipitis strains. Appl. and Environm. Microbiol. 52: 320-324.

MACKO, V., A. NOVACHY and M.A. STANHMANN. 1967. Protein and enzyme patterns from uredispores of Puccinia graminis var. tritici. Phytopathol. Z. 58: 122-127.

MAYER, A.M. and E. HAREL. 1970. Polyphenol oxidases in plants. Phytochemistry 18: 193-215.

MILTON, J.M., W.G. ROGERS and I. ISAAC. 1971. Application of acrylamide gel electrophoresis of soluble fungal proteins to taxonomy of Verticillium species. Trans. Brit. Mycol. Soc. 56: 61-65.

O'FARRELL, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250: 4007-4021.

PAREKH, S.R., S. YU and M. WAYMAN. 1986. Adaption of Candida shehatae and Pichia stipitis to wood hydrolysates for increased ethanol production. Appl. Microbiol. Biotechnol. 25:300-304.

SHAW, C.R. and R. PRASAD. 1970. Starch electrophoresis of enzymes - a compilation of recipes. Biochem. Genet. 4: 297-320.

SICILIANO, M.J. and C.R. SHAW. 1976. Separation and visualization of enzymes on gels. In Chromatographic and electrophoretic techniques. Edited by I. Smith. Heinemann, London, pp. 185-209.

STIPES, R.J. 1970. Comparative mycelial protein and enzyme patterns in four species of Ceratocystis. Mycologia 62: 987-995.

WAYMAN, M. and S.R. PAREKH. 1985. Ethanol and sugar tolerance of Candida shehatae. Biotechnol. Lett. 7(12): 909-912.

WAYMAN, M. and S.T. TSUYUKI. 1986. Fermentation of xylose to ethanol by Candida shehatae. Biotechnol. Bioeng. Symp. No. 15 (1985). John Wiley & Sons Inc., NY, pp. 167-177.

YU, S. and M. WAYMAN. 1986. Fermentation of spent sulphite liquor to butanol and ethanol. J. Pulp Paper Sci. 12(3): J72-77.

YU, S., M. WAYMAN and S.R. PAREKH. 1986. Fermentation to ethanol of pentose-containing spent sulphite liquor. Biotechnol. Bioeng. (in press).

Explanation of Figures

Figure 1. Isoeznyme banding patterns for alcohol dehydrogenase (a), aldolase (b), esterase (c), glucose-6-phosphate dehydrogenase (d), peroxidase (h), phosphoglucomutase (i), 6-phosphogluconate dehydrogenase (j), succinate dehydrogenase (k) and tetrozolium oxidase (l) of Candida shehatae (1), C. shehatae R (2), Pichia stipitis (3) and P. stipitis R (4). The adapted strains exhibited similar banding patterns for all the enzymes tested.

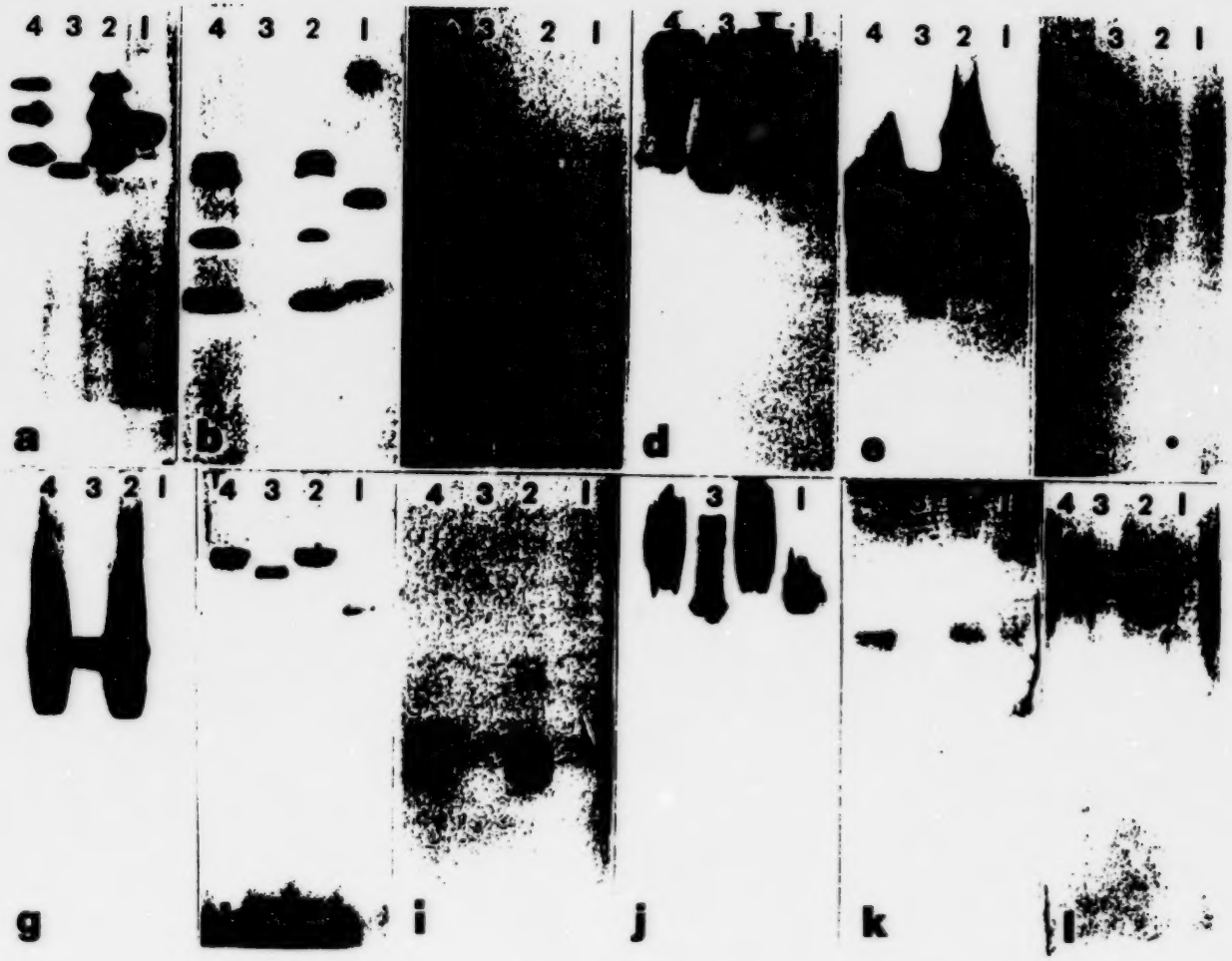
Figure 2. Electrophoretic soluble protein patterns on SDS polyacrylamide gradient gel (a) and NON-SDS polyacrylamide gel (b) of Candida shehatae (1), C. shehatae R (2), Pichia stipitis (3) and P. stipitis R (4).

Figure 3. Isoelectrofocusing of soluble proteins extracted from the vegetative cells of Candida shehatae (1), C. shehatae R (2), Pichia stipitis (3) and P. stipitis R (4). The two recycled strains have a very similar banding patterns while the two starting strains show their own characteristic protein profiles. The inter-electrode distance is 11 cm.

Figure 4. Two-dimensional polypeptide mapping of soluble proteins extracted from the vegetative cells of the two starting strains, Candida shehatae (A) and Pichia stipitis (B). C. shehatae can be distinguished from P. stipitis by the

presence of protein spots 1, 2, 3, 4, 5 and 6. P. stipitis can be recognized by protein spots a, b, c, d and e.

Figure 5. Two-dimensional polypeptide mapping of soluble proteins extracted from the vegetative cells of the two adapted strains, Candida shehatae R (C) and Pichia stipitis R (D). Note the similarity in the protein pattern of these two strains. Each adapted strain possesses its own characteristic protein spots (arrows).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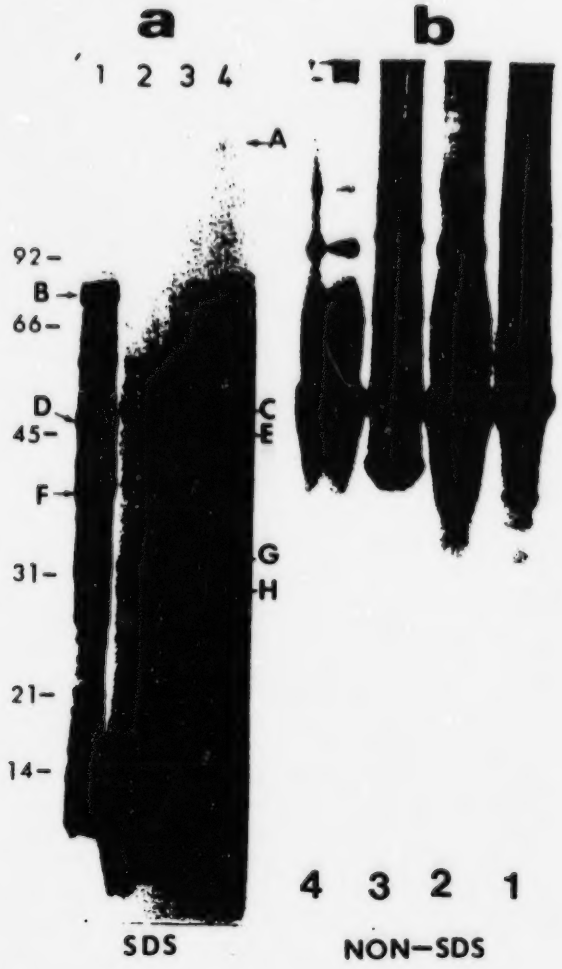
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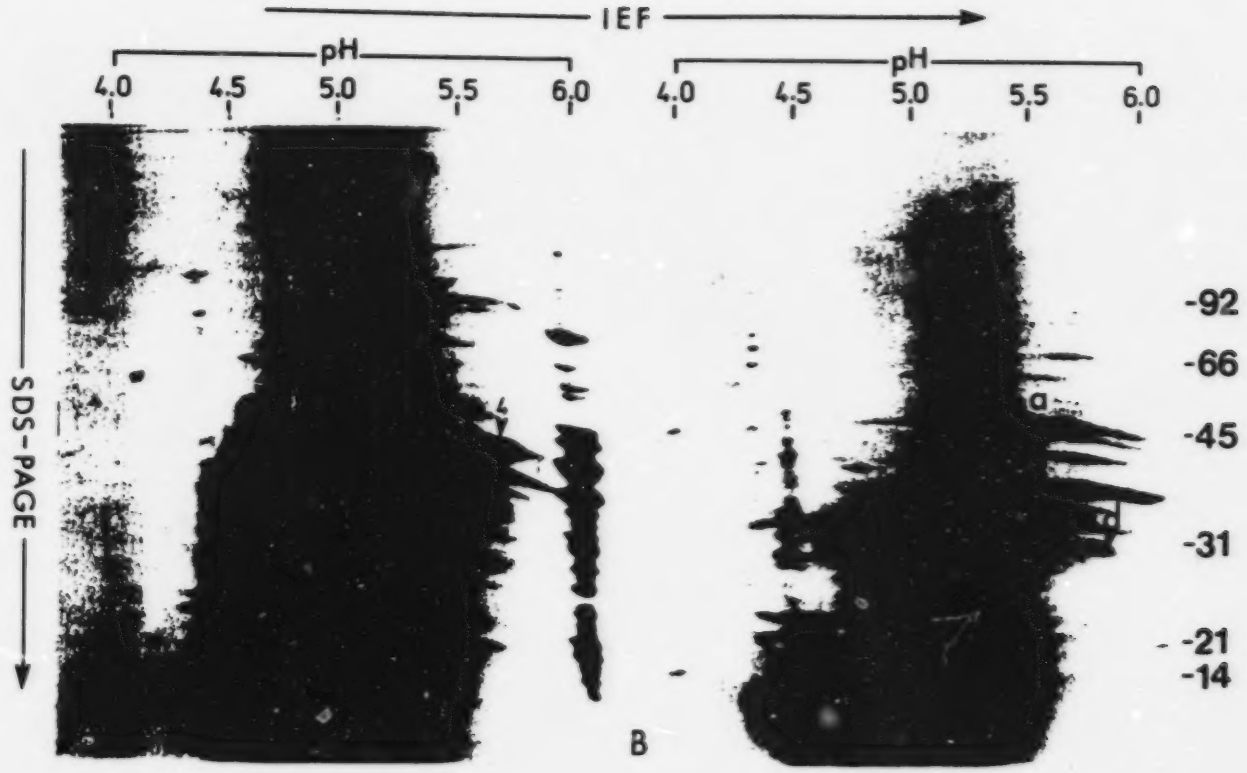
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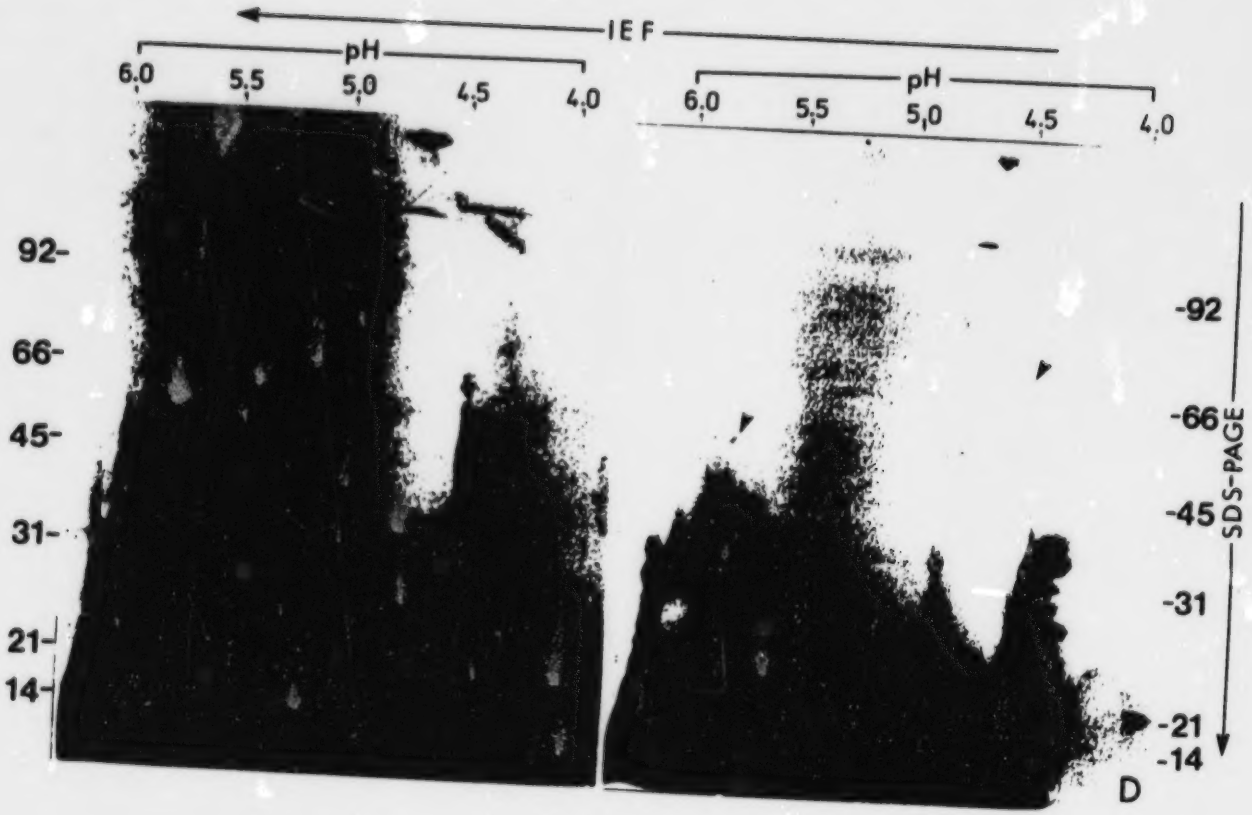
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Performance of a novel continuous dynamic immobilized cell bioreactor in ethanolic fermentation

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SUMMARY

Continuous ethanol production from glucose, sucrose, cane molasses and corn wet milling waste by cells of Saccharomyces cerevisiae and on first three substrates by cells of Pichia stipitis (R) was investigated. The cells were immobilized on rotating fiber discs by calcium alginate gel in a 2 l novel continuous dynamic immobilized cell (CDIC) bioreactor. Ethanol productivity from these substrates was enhanced by creating more available space between fiber discs to facilitate removal of evolved CO_2 entrapped among the fiber discs. Glucose and sucrose at about 55 gl^{-1} were fermented in 1.6 h with about 90% substrate utilization and waste water from corn wet milling process with 115 gl^{-1} sugars was fermented completely in 2.5 h by S. cerevisiae loaded at 56 gl^{-1} cell density. Cane molasses containing 195 gl^{-1} total sugars was fermented in 3.6 h (90% substrate utilization) by S. cerevisiae with a maximum end product of 83 gl^{-1} ethanol (10.6% by volume) and an ethanol productivity of 23 $gl^{-1}h^{-1}$. The bioreactor was run continuously with cane molasses containing 195 gl^{-1} total sugars at a fixed flow rate of 0.55 lh^{-1} (dilution rate = 0.27 h^{-1}) for 15 days, during which there was a loss of 8% of the original activity. The half life of the bioreactor was estimated to

be about 98 days. The performance of P. stipitis (R) was found to be slightly inferior to S. cerevisiae. The merits and the potentials of this fermenter for ethanol production is discussed.

Keywords: Ethanol fermentation, whole cell immobilization, bioreactor, high ethanol productivity, continuous dynamic immobilized cell fermenter.

INTRODUCTION

Recently there has been worldwide interest in the production of ethanol by fermentation as an alternative to chemical feed stock and as a gasoline extender. Most of the ethanol fermentation practised today is by batch methods. The main drawbacks of the batch fermentations are the long, continual start up and shut down operation, high labour cost, large fermenter volume requirements and low productivity. Improving productivity of the fermenter requires the development of a "high rate" process with reduced fermenter and operating costs, which essentially means a) continuous operations, b) high cell density in the bioreactor, c) minimized residence time, d) distillation feed containing a high concentration of ethanol, and e) rapid and continuous removal of inhibitory end products, especially CO_2 gas. Several new technologies have been developed and proposed to meet the above goals including vacuum fermenters[1], cell recycling[2], hollow fiber membrane reactors with recycling[3] and immobilization of whole cells biocatalyst in polymeric gels[4-6]. The use of vacuum-assisted fermentation with recycling involves the high cost of centrifuges, while the use of ultrafiltration membranes, apart from being costly, have many mechanical problems. The use of viable whole cells immobilized in solid gel matrices as beads has gained wide spread attention and has been studied in packed bed[7] and fluidized-bed reactors[8]. However, due to the diffusional resistance of substrate or

product and the requirement of rapid removal of CO_2 from the reactor, together with microbial viability for long term production of ethanol, these systems have relatively low efficiency and find limited applications.

Robert Clyde[9] developed a rotating fiber disc fermenter suitable for carrying out a 3-phase reaction involving liquid substrate, solid immobilized cell biocatalyst and CO_2 gas. In his study, bacterial cells, Zymomonas mobilis, were trapped in porous fiber discs by physical adsorption and rotated in a bioreactor containing fermentable substrate. Fiber discs offered a large surface area for immobilization and the rotation resulted in better mixing between the 3-phases and rapid removal of evolved CO_2 from the liquid phase. However this reactor has serious short comings: a) the amount of cells loaded by simple adsorption in the fiber discs is low, and b) the rotation of the fiber discs results in the detachment of cells from the surface of the fiber discs and consequently substantial loss of microorganisms from the reactor. The end results are high investment costs for centrifuges to separate cells from the distillation stream and decreased efficiency of the reactor during long periods of operation. Also, no literature data is available on the performance of this system with immobilized yeasts, the microbes utilized today in the fermentation ethanol industry. These considerations prompted us

to modify the reactor system and study the use of rotating fiber discs with immobilized yeasts.

To maintain high cell density in the reactor and to overcome the negative effect of cell loss, the authors have introduced the technique of lightly immobilizing the yeast cells adsorbed in the fiber discs with low concentrations of calcium alginate gel. By such a procedure yeast cells are trapped and efficiently immobilized and bound to the supports, forming a thin film on the surface of the fiber discs. Such a method maintains high cell density without causing excessive cell leakage when the discs are rotated, and the thin film of gel coated cells offers least mass transfer resistance through the porous fiber discs. The present paper describes the performance of this modified continuous dynamic immobilized cell (CDIC) bioreactor with respect to ethanol production from saccharine substrates normally adopted in industrial practice and highlights the merits and potential of CDIC bioreactor for industrial application.

MATERIALS AND METHODS

Organisms

Saccharomyces cerevisiae used was an industrially dried bakers yeast (Fleischmanns) obtained from the local market. Pichia stipitis (R) was a strain derived from Pichia stipitis CBS 5776 recycled several times in wood hydrolysate[10]. This strain was used in the

present study for comparison with S. cerevisiae since it has the ability to ferment rapidly sugars derived from lignocellulosic feed stock[11].

Medium

The feed medium consisted of reducing sugars at concentrations ranging from 45 to 200 gl^{-1} from various saccharine substrates, supplemented with 1 gl^{-1} yeast extract, and 1 gl^{-1} NH_4Cl at pH 5.0. Urea was added separately at 0.1 gl^{-1}. Cane molasses was obtained from Canada West Indies Molasses Co., Mississauga, Ontario, and acid liquified corn wet milling waste (12% dry solids) from a nearby 30,000 bushels/day corn wet milling plant. Cane molasses was diluted to required sugar strengths with tap water, pH adjusted to 5 and steam sterilized. Corn wet milling waste water was enzymatically saccharified at pH 4.8 with 0.1% (w/v) glucoamylase (Novo) for 6 h at 50°C. The amounts of various nutrients added to these substrates were the same as described above. D-glucose and sucrose were obtained locally.

Bioreactor

A schematic diagram of the experimental set-up is shown in Figure 1. The reactor was made up of a stainless steel cylinder, 35 cm long and 11 cm in diameter, in which fiber discs (9.5 cm in diameter) were mounted on a central stainless steel shaft (0.5 cm in diameter). The fiber discs which were randomly perforated to increase flow of medium

through the fermenter, were rotated by a fixed speed motor connected to the shaft at 40 rev min^{-1}. In one configuration ("close disc spacing") the discs were 10/cm, while in an "open spacing" configuration, these were 5 discs/cm. Between the motor and the reactor wall a rubber ring was used to prevent leakage. The upper transparent removable cover, having orifices for feed inlet and 3 chimneys for CO_2 outlet, was tightly connected to the body of the fermenter. The outlet gas lines were connected to 3 condensers which removed moisture and volatile components. Chilled water (4°C) was circulated through the condensers. The reactor temperature was controlled by placing it in a constant temperature incubator chamber. The bioreactor was placed slightly inclined to the horizontal, the outlet being above the inlet.

Cell immobilization

Cells of P. stipitis (R) for immobilization were grown in 4 l Erlenmeyer flasks with 2 l of medium of the following composition (gl^{-1}): D-glucose 40, yeast extract 3, malt extract 3, peptone 5 at pH 5.0 and grown on a rotating shaker at 150 rpm. The cells were harvested in the late exponential phase of growth and concentrated by centrifugation at 1000 rpm (164 g) for 10 min. The CDIC bioreactor together with the fiber discs were sterilized with 5% formaldehyde solution and washed several times with hot distilled water. Sodium alginate (Fisher Scientific) was used as the cell entrapment material.

Concentrated free cells were suspended in 0.85% NaCl to which 0.1% sodium alginate was added and mixed thoroughly to form a thick uniform suspension. The resulting slurry (at 30°C) was introduced into the previously sterilized reactor and the fiber discs rotated by the motor. During this period, the alginate coated yeast cells were physically entrapped in and on the fiber discs. After 4 hrs, a 2% $CaCl_2$ solution was added, causing the cells to be strongly bound in the form of a thin film of calcium alginate on the surface of disc support. The film was allowed to harden in $CaCl_2$ solution for 6 hrs. Untrapped cells were leached out by washing the reactor with sterile saline, until the effluent was clear.

Bioreactor operating procedure

The liquid phase volume in the fermenter was 90% of the total bioreactor volume of 2.2 l. The cells immobilized on the fiber discs were rotated constantly at 40 rev min^{-1}. A positive displacement peristaltic pump was used to vary the liquid feed flow rate. The flow rate of the medium was determined by timing the volume collected at the overflow outlet of the bioreactor, and the dilution rate of the reactor was calculated from the reciprocal of the liquid residence time in the fermenter[12]. The pH was not controlled during the experiment because pH varied only slightly from the initial value of 5 to a final value of 4.8. This slight change in pH (in the short residence time) was considered to have no significant effect on the

fermentation rate of the yeasts. The continuous operations were conducted at different volumetric flow rates and at each flow rate the system was considered to have reached steady state once 5 fermenter volumes had passed, and when ethanol, reducing sugars and free cell concentrations remained constant as evidenced by 3 successive samples. Only steady state data are reported.

Analysis

Ethanol was determined by GC using n-propanol as the internal standard[13]. D-glucose was measured with a glucose analyser (YSI - Yellow Springs Instruments). Total reducing sugars were analysed by Nelson-Somogyi method[14]. Yeast cell dry weight immobilized per unit reactor volume (gl^{-1}) was measured optically at 660 nm, using a Spectronic 20 (Bausch and Lomb), and converted to units of gl^{-1} with the aid of a calibration curve[15].

RESULTS

Tables 1 and 2 gather the main results of each run with the different substrates and summarize the ethanol produced and % substrate utilized from each experimental run conducted with varying flow rates. Since most of the ethanol producing industries practise fermentations leading to 85% and greater substrate conversions[16,17], only those results are reported here in which continuous operations leading to over 85% substrate utilization occurred, and from the data

their least mean residence time and ethanol productivity at that flow rate were calculated.

Table 1 shows S. cerevisiae to ferment sucrose and glucose in less than 2 hours with 86 and 94% substrate utilization respectively when the close disc spacing was employed. The yield was 0.47 g ethanol/g sugar consumed with these two substrates at 59 and 50 gl^{-1}. Ethanol concentration in the effluent was over 22.4 gl^{-1}, or above 3% by volume. Under the same conditions, cane molasses diluted to a sugar concentration of 100 gl^{-1} was completely fermented in 3 hours with high productivity, ethanol concentration being 5.8% by volume.

Under the same conditions, that is close disc spacing, Pichia stipitis (R) was able to ferment sucrose, glucose and cane molasses to 95 to 84% substrate utilization and good yields of ethanol based on sugar consumed. However the retention times were longer in each case than experienced with S. cerevisiae, being 3.0 and 2.6 h for sucrose and glucose, respectively, and 6 h for cane molasses.

During the operation of this fermenter, violent evolution of CO_2 was always observed and most of the CO_2 evolved appeared to be trapped among the fiber discs. This resulted in high CO_2 gas holdup and creation of dead spaces, thereby limiting the performance of the reactor. To overcome this, about 50% of the discs were removed, and the remaining fiber discs reassembled leaving 1 mm space with the aid of rubber washers between the discs. The fiber discs in this modified

"open spacing" configuration could be reused and reloaded after the cells were removed by washing away with hot phosphate buffer.

When open disc spacing was employed, fermentation with S. cerevisiae proceeded more rapidly than with close disc spacing, retention times for sucrose and glucose being 1.6 h, with excellent substrate utilization and almost theoretical yield of ethanol. In this set of runs, the cane molasses was diluted only to 195 gl^{-1} sugars. Excellent, rapid fermentation was observed, retention time being 3.6 h, sugar utilization being 90% and yield 0.48 g ethanol/g sugar consumed. Ethanol concentration in the fermenter product was 10.6 vol %. Fermentation with corn wet milling waste having 115 gl^{-1} fermentable sugars was completely fermented to ethanol with yields close to theoretical and productivity of 22.4 $gl^{-1}h^{-1}$. As found with close disc spacing, fermentation with P. stipitis (R) was somewhat slower, about 2 h retention time for sucrose and glucose, and about 5 h for cane molasses. Substrate utilization and ethanol yields were about the same as those observed with S. cerevisiae.

Operational stability

In order to determine the operational stability of the CDIC fermenter, it was operated continuously at a fixed flow rate of 0.55 l h^{-1} (dilution rate = 0.27 h^{-1}, 3.6 h mean residence time) with S. cerevisiae loaded at 56 gl^{-1} cell density (based on total reactor volume) for a period of 15 days on a cane molasses medium having about

195 gl^{-1} sugars. Figure 2 shows the plots of ethanol, total reducing sugars, free cell concentration and pH of the effluent as functions of operating time. The data show that during the first 10 days, the values of ethanol and sugars in the effluent remained almost constant with no change in the steady state, but beyond 10 days there was a steady decrease in ethanol concentration with corresponding increase in unutilized substrate. The effluent biomass concentration increased from 0.22 gl^{-1} to 0.57 gl^{-1} dry weight cells over the 15 day period. There was not much change in pH values. From the data presented in Figure 2, various kinetic parameters such as volumetric ethanol productivity, percent feed sugars utilized, and ethanol yields were calculated and plotted as functions of time in Figure 3. The value of volumetric ethanol productivity declined from 23 $gl^{-1}h^{-1}$ at 91% substrate utilization to 20 $gl^{-1}h^{-1}$ at 83% substrate utilization respectively in 15 days. At this time the ethanol yield fell from 0.48 to 0.45 g ethanol/g sugar consumed which represents 88% yield of theoretical. No contamination was noticed during the continuous fermentation operation and only a small amount of sludge from molasses adhered to the discs and the remaining sludge was not retained during operation.

DISCUSSION

Ethanol productivity of about 23 $g^{-1}h^{-1}$ obtained from cane molasses and corn wet milling waste water in the CDIC fermenter is higher and its performance better than those reported for continuous culture[18], tower fermenters[19], rotating packed drum reactor[6] and immobilized cells[20]. It also compares favourably with pilot plant operation tested in Japan with calcium alginate immobilized beads[21] and polyethylene glycol photo crossed-linked resins[22] as carrier. Although ethanol productivities as high as 100-120 gl^{-1} have been reported with fermenters coupled with an external ultrafiltration unit[2] or with membrane reactors[3], scale up of such technology would be impractical with industrial substrates such as molasses, cane juice, and hydrolysed starch, and lignocellulosic materials containing high solids which would clog the membranes and would need special sanitation care.

The initial activity of the CDIC bioreactor decreased by about 8% during 15 days of continuous operation. On the basis of this data, the estimated "half life" of the reactor operated at 0.55 lh^{-1}, dilution rate = 0.27 h^{-1} was 98 days. Since the reactor activity remained constant for the initial 10 days and declined slowly there after, to maintain continuous prolonged activity of the reactor, the cells in the reactor need reviving every 10 days. To achieve this, O_2, growth factors, sterols and unsaturated fatty acids are normally

added to the feed substrate. The procedure results in maintaining continuous growth and viability with prolonged catalytic activity of yeast cells in immobilized bioreactors[21].

Table 3 shows the advantages of using the CDIC reactor for ethanol production as compared to conventional practical fermentation. The data shows that the CDIC fermenter has productivity of alcohol 12-18 times higher than conventional batch fermentation and enables the fermentation to be completed in a few hours compared to 2-3 days required in batch processes. The process also represent a remarkably lowered initial investment and operating cost. The newly designed CDIC reactor described shows great potential for industrial application, since it is able to handle any sugar solution presently industrially adopted and which may be obtained from biomass resources in future. It also offers the following merits:

- capable of high cell loading and maintaining this at high levels
- sterilizable and reuseable supports
- unique procedure in which both immobilization and fermentation are carried out in the same vessel, with low chance of contamination. This also decreases the initial investment costs
- easy elimination of CO_2 and volatile gases
- high ethanol output

- maintenance of high viability of yeast cells and
- minimal product inhibition due to low retention time.

CONCLUSION

Several new technologies for continuous ethanol fermentation have been proposed in recent years. The newly designed CDIC fermenter showed great promise of fermenting saccharine substrates rapidly and efficiently. This reactor has industrial potential and could be adopted for economical production of ethanol on a continuous basis.

ACKNOWLEDGEMENTS

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REFERENCES

1. Cysewski, G.R. and Wilke, C.R. Biotechnol. Bioeng. 1977, 19, 1125-1143

2. Rogers, P.L., Lee, K.J. and Tribe, D.E. Process Biochem. 1980, 15, 7-11

3. Cheryn, M. and Mahaia, M.A. Process Biochem. 1984, 19, 204-208

4. Cheetham, P.S.J., Blunt, K.W. and Bucke, C. Biotechnol. Bioeng. 1979, 21, 2155-2168

5. Chang, H.N., Joo, I.S. and Ghim, Y.S. Biotechnol. Lett. 1984, 6, 487-492

6. Lee, C.W. and Chang, H.N. Enzyme Microbiol. Technol. 1985, 7, 561-563

7. William, D. and Munnecke, D.M. Biotechnol. Bioeng. 1981, 23, 1813-1825

8. Nagashima, M., Azuma, M., Noguchi, S., Inuzuka, K. and Samejima, H. Biotechnol. Bioeng. 1984, 26, 992-997

9. Robert Clyde, U.S. Patent, No. 4,407,954 1983, on "Fiber Fermenter"

10. Parekh, S.R., Yu, S. and Wayman, M. Eur. J. Appl. Microbiol. Biotechnol. 1986, in press

11. Wayman, M., Parekh, S., Chornet, E. and Overend, R.P. Biotechnol. Letters 1986, in press

12. Krouwel, P.G. and Kossen, N.W.F. Biotechnol. Bioeng. 1981, 23, 651

13. Wayman, M. and Tsuyuki, S.T. Biotechnol. Bioeng. Symp. 1985, 15, 167-177

14. Somogyi, M. J. Biol. Chem. 1952, 195, 19-23

15. Yu, S., Wayman, M. and Parekh, S. Biotechnol. Bioeng. 1986, in press

16. Jakson, E.A. Process Biochem. 1976, 11, 29-30

17. Kolot, F.B. Process Biochem. 1980, 15, 2-8

18. Ghose, T.K., Tyagki, R.D. Biotechnol. Bioeng. 1979, 21, 1387

19. Netto, C.B., Destruchaut, A. and Goma, G. Biotechnol. Lett. 1985, 7, 355-360

20. Ghose, T.K. and Bandhopadhyay, K.K. Biotechnol. Bioeng. 1980, 22, 149

21. Technical report on "Continuous Alcohol Fermentation with Immobilized Growing Yeast", 1983, Kyowa Hakko Kogyo, Co. Ltd., Japan

22. Technical report on "Continuous Alcoholic Fermentation by Immobilized Yeast Cells", 1983, J.G.C. Corp., Japan

Table 1 Performance of continuous dynamic immobilized cell fermenter - close disc spacing.

Substrate	Sugar concentration (gl^{-1})	Sugar utilization (%)	Ethanol in effluent (gl^{-1})	Ethanol productivity ($gl^{-1}h^{-1}$)	Retention time (h)	Yield g ethanol/ g sugar consumed	Cell loading in reactor (gl^{-1})	Cell in effluent (gl^{-1})
			Saccharomyces cerevisiae					
Sucrose	59	86	24	12.6	1.9	0.47	56	0.18
Glucose	50	94	22.4	12.4	1.8	0.47	56	0.20
Cane molasses	100	93	45	14.5	3	0.48	56	0.26
			Pichia stipitis (R)					
Sucrose	56	95	25.6	8.5	3.0	0.48	56	0.22
Glucose	50	95	22.9	8.8	2.6	0.49	56	0.21
Cane molasses	114	84	43	7	6.0	0.45	56	0.24

Table 2 Performance of continuous dynamic immobilized cell fermenter - open disc spacing.

Substrate	Sugar concentration (gl^{-1})	Sugar utilization (%)	Ethanol in effluent (gl^{-1})	Ethanol productivity ($gl^{-1}h^{-1}$)	Retention time (h)	Yield g ethanol/ g sugar consumed	Cell in effluent (gl^{-1})	Cell loading in reactor (gl^{-1})
			Saccharomyces cerevisiae					
Sucrose	55	88	24.2	15.2	1.6	0.50	0.18	56
Glucose	54	90	24.3	15.2	1.6	0.50	0.21	56
Cane molasses	195	90	83.0	23	3.6	0.48	0.22	56
Corn wet milling waste	115	98	56	22.4	2.5	0.50	0.22	56
			Pichia stipitis (R)					
Sucrose	54	86	22.8	11.4	2.0	0.49	0.18	56
Glucose	50	92	22	11	2.0	0.48	0.21	56
Cane molasses	190	86	78	15.6	5	0.48	0.22	56

Table 3

Features of continuous alcoholic fermentation in a CDIC bioreactor by *S. cerevisiae*

Process and features	Conventional process	Mille-Bionet process[22]	Immobilized yeast cells in CDIC reactor	
			Present results	Future targets
Fermentation Type	Batch	Batch	Continuous	Continuous
Cell concentration (gl^{-1})	2-3	9.6	56	80-100
Final ethanol concentration (gl^{-1})	100-110	55-80	85	90-110
Yields from sugars (%)	85-86	85-86	90	95
Ethanol productivity ($gl^{-1}h^{-1}$)	1.3-2.0	2-4	23	30-40
Fermentation time (h)	70-76	15-25	2-6	2-3

CAPTIONS TO FIGURES

Figure 1. Schematic diagram of experimental set up
1, rotating fiber disc fermenter (2.2 1); 2, constant speed motor; 3, condenser; 4, thermometer; 5, feed bottle (20 1); 6, product bottle (20 1); 7, peristaltic pump; 8, stand; 9, central shaft; 10, fiber discs.

Figure 2. Performance of CDIC fermenter with 19.5% molasses sugar as a function of time at constant feed flow rate of 0.55 $1h^{-1}$ ($D = 0.27$ h^{-1}); (●) ethanol; (△) total reducing sugars; (○) pH; (□) free cell concentration.

Figure 3. Stability of CDIC bioreactor with 19.5% molasses sugar fermentation as a function of time at constant feed flow rate, 0.55 $1h^{-1}$, (●) % sugar utilized; (○) ethanol yield; (△) volumetric ethanol productivity.

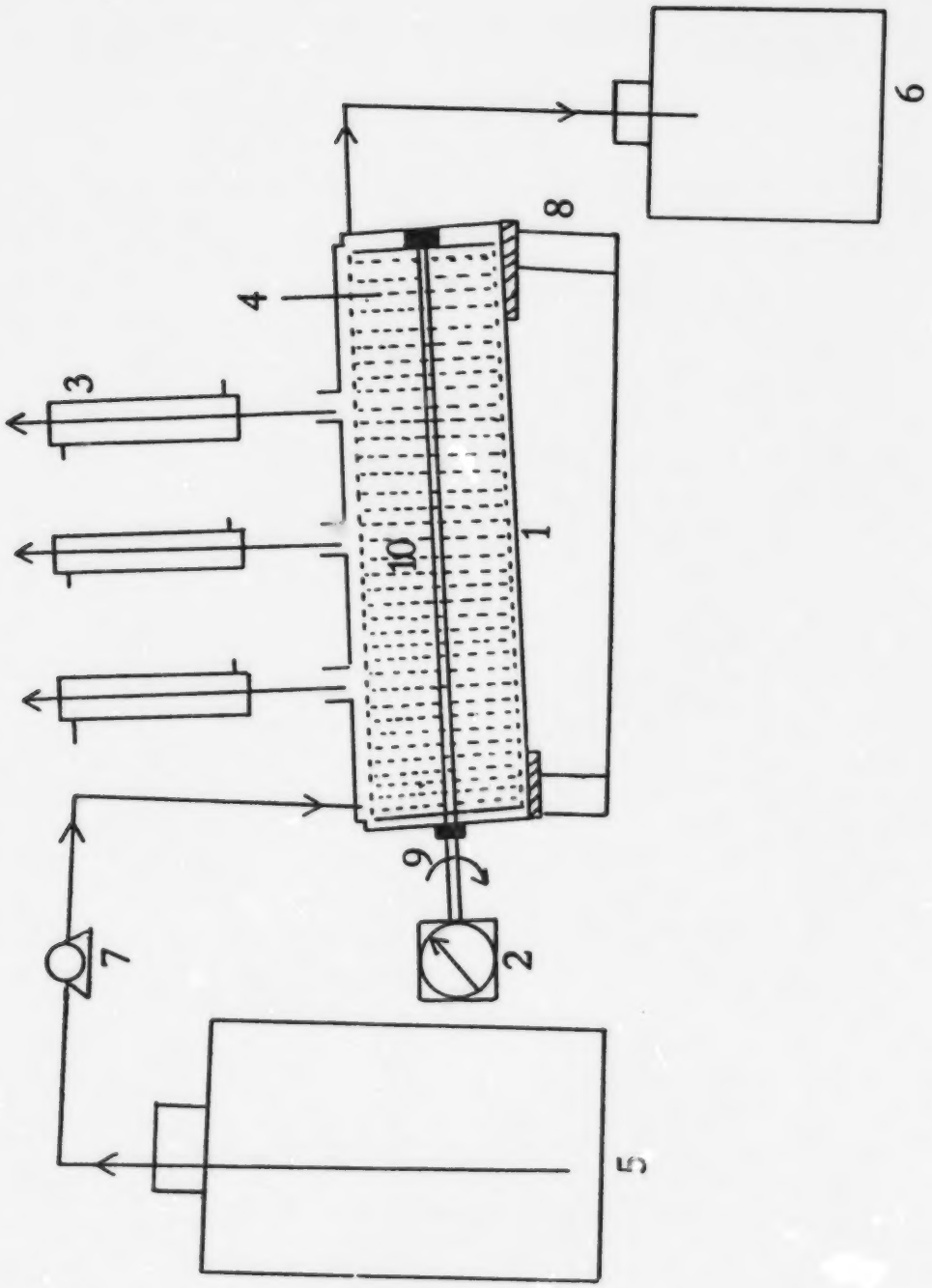


Fig 2

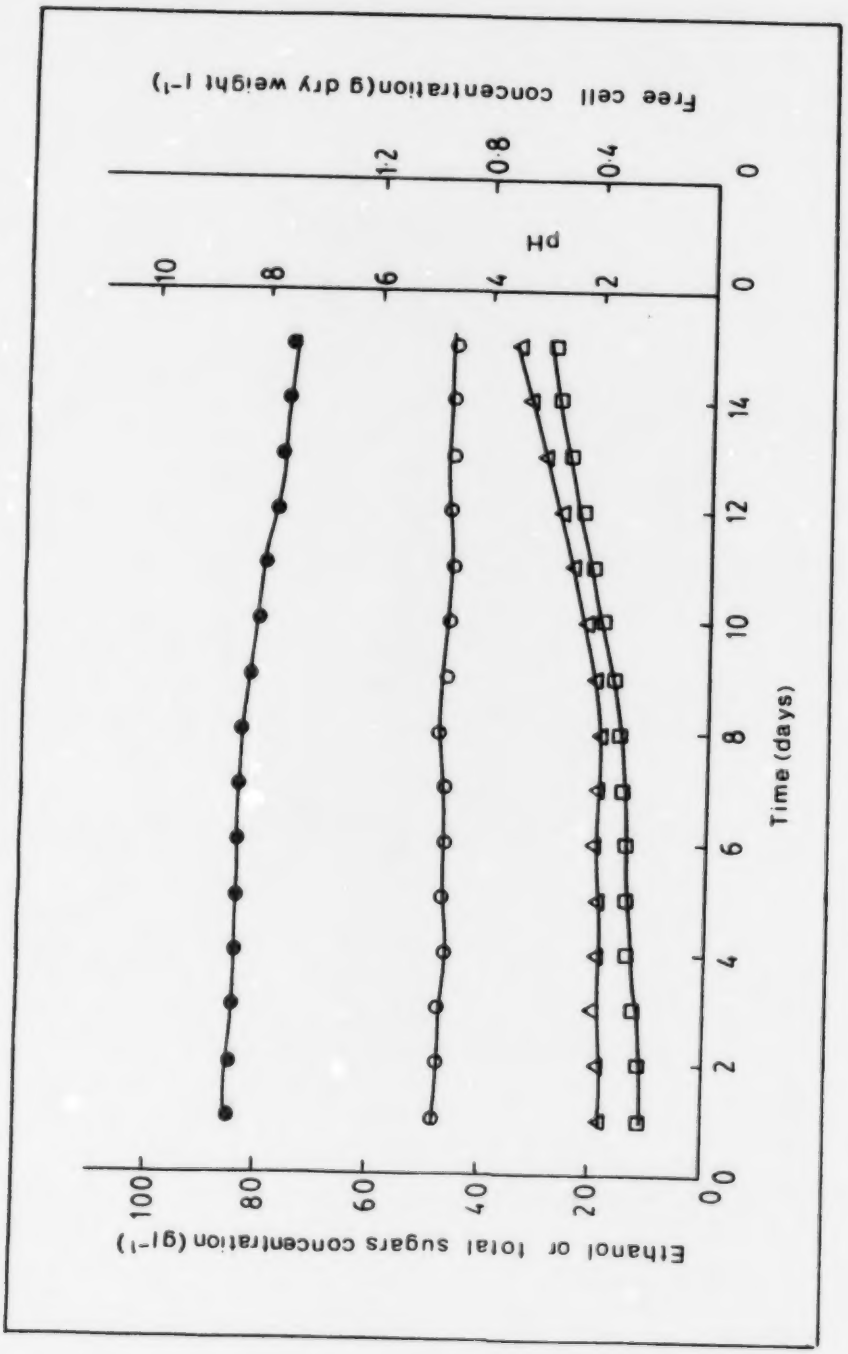
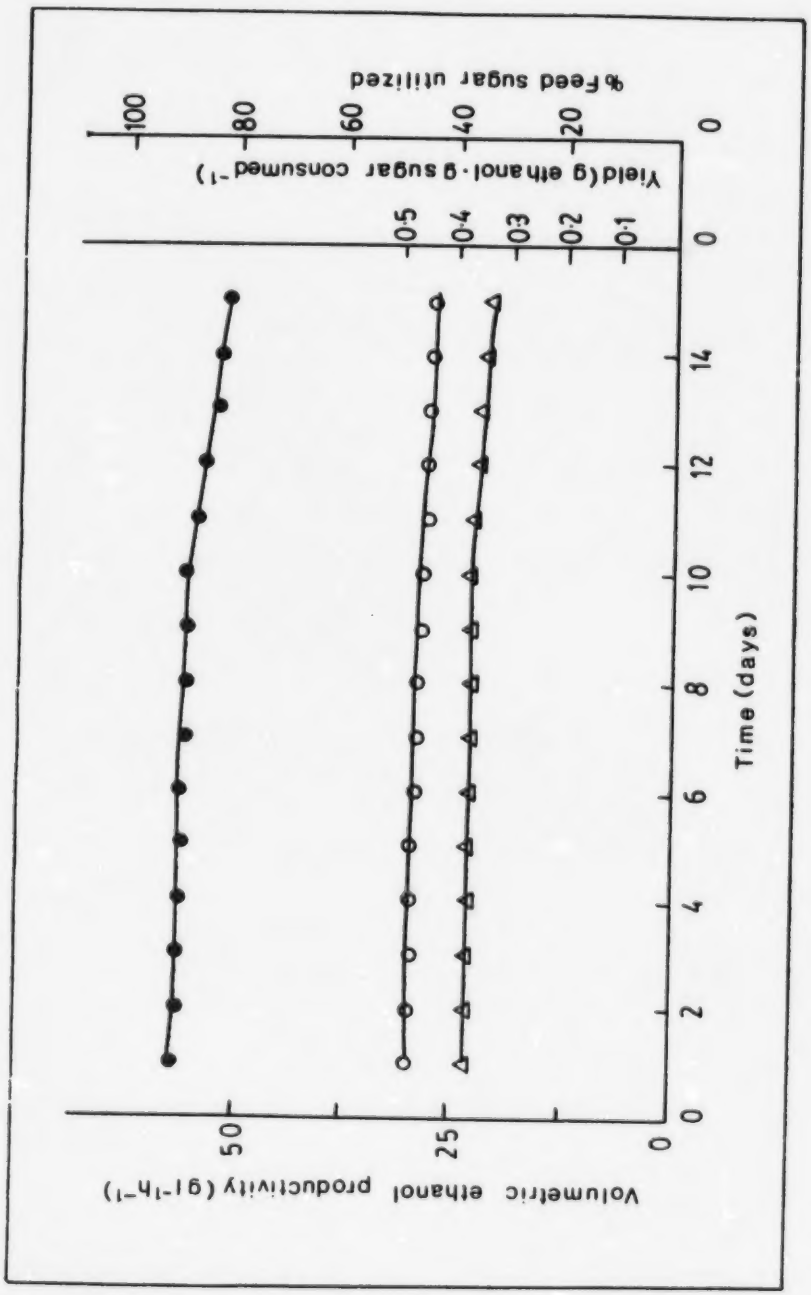

Ethanol or total sugars concentration (gl⁻¹)
pH
Free cell concentration(g dry weight l⁻¹)
Time (days)

Fig 2


Volumetric ethanol productivity (gl⁻¹h⁻¹)
50
25
0
0
2
4
6
8
10
12
14
Time (days)
Yield (g ethanol · g sugar consumed⁻¹)
0·5
0·4
0·3
0·2
0·1
0
% Feed sugar utilized
100
80
60
40
20
0

Ethanol fermentation by Pichia stipitis of combined pentose and hexose sugars from lignocellulosics prehydrolysed by SO_2 and enzymatically saccharified

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SYNOPSIS

Lignocellulosic biomass, namely pine, aspen and corn stover, were prehydrolysed using SO_2 as the hydrolysing catalyst. The prehydrolysis residues were directly saccharified, without washing, by cellulolytic enzymes, whereby about 90% of theoretical yields of sugars were obtained. Fermentation of the hydrolysates containing both the hemicellulose and cellulose sugars by a pentose fermenting yeast, Pichia stipitis R, gave yields of 372, 346 and 388 ℓ ethanol/tonne (dry weight) from pine, aspen and corn stover respectively. The technical aspects of the SO_2 catalysed prehydrolysis and enzymatic saccharification stages and the advantages of fermentation by pentose fermenting yeasts are discussed.

INTRODUCTION

The phase-out of lead in gasoline as octane boosters has provided new applications and markets for alcohols to enhance octane ratings. In this respect, ethyl alcohol is gaining increased importance as an engine fuel since it is easily blended with gasoline[1]. Presently most fermentatively produced fuel alcohol plants in the world are based on corn or on cane molasses[2]. Corn is nutrition for both humans and animals; and this use has raised concern about driving engines based on human food or animal feed. An attractive alternative appears to be the biotechnological conversion to power alcohols of widely available renewable lignocellulosic biomass such as waste wood and agricultural residues, which can serve as cheap sources of fermentable sugars[3-6].

In the past, factors which have delayed immediate application of cellulosics for fuel supply have been: a) inability to hydrolyse the biomass completely to generate high yields of fermentable sugars[7], and b) failure to ferment the pentose sugar xylose of the hemicellulose fraction of lignocellulose, which comprises as much as 30-40% of the plant material. This was considered unfermentable and in most cases wasted[6]. This scene, however, has been altered with the availability of new processes and equipment for treatment of biomass to recover high yields of sugars[8], and the fairly recent discovery of yeasts such as Pachysolen tannophilus, Candida shehatae and Pichia stipitis, capable of fermenting pentoses directly to ethanol[9-11].

Among the pentose fermenting yeasts, Pichia stipitis offers advantages over other yeasts for direct fermentation of pentose containing

liquors because this yeast exhibits efficient ethanol fermentation from xylose, cellobiose[12] and other wood sugars, and apparently it produces no byproduct, xylitol[11]. Apart from this, improved strains of P. stipitis and C. shehatae are reported[13] having enhanced ability in utilization and fermentation of pentose-containing lignocellulosic hydrolysates and are tolerant of and resistant to toxic substances that may be generated from these feed stocks. Such advances have sparked renewed interest in power alcohols production by cellulolytic fermentation and have led France[14] and Japan[15] to establish large pilot plants.

Because of the heterogeneous nature of lignocellulosic biomass, there is no hydrolytic process known capable of converting these raw materials in a single stage of reaction to good yields of sugars. Such processes therefore rely on at least 2 stages of hydrolysis. In the pretreatment or first stage, the hemicellulose is hydrolysed and the cellulose is made more susceptible to hydrolytic agents such as mineral acids or enzymes, and in the second stage, the cellulose is hydrolysed to fermentable sugars. Pretreatment using steam (autohydrolysis) has been found to be highly effective for hemicellulose hydrolysis of deciduous wood, such as poplar, and agricultural crops[16,17]. This method, however, has not found immediate application in pretreatment of coniferous woods such as pine, spruce and hemlock, which are the main residues of the forest industry. Recently, the merits of using steam pretreatment in the presence of SO_2 catalyst (prehydrolysis) to produce sugars from various lignocellulosic biomass, including pine, aspen and corn cobs, were reported[16], with encouraging results. This led us to investigate the

application of SO_2 prehydrolysis to produce substrates for fuel ethanol production.

The present paper reports the results of the enzymatic saccharification of these feedstocks following prehydrolysis by SO_2, and the application of a pentose fermenting yeast, Pichia stipitis, to ethanolic fermentation of the hydrolysates containing both hemicellulose and cellulose sugars.

MATERIALS AND METHODS

Raw materials:

The raw materials used in the present investigation were soft wood, pine from Southern Ontario and mixed coniferous woods from the Eastern Townships of Quebec, aspen poplar chips from Northern Ontario and corn stover from a nearby farm. These materials were analysed as described earlier[16], and their chemical composition is given in Table 1.

Prehydrolysis with SO_2:

Samples of about 100 g (dry weight) of aspen and corn stover were treated in a laboratory 7 ℓ direct steam batch reactor, with saturated steam in the presence of a small amount of water, the liquid/solid ratio being about 2:1 and 3% SO_2 on dry fiber. The time and temperature for prehydrolysis were 30 minutes at 75 psig (160°C). The results of prehydrolysis of aspen and corn stover are shown in Table 2 and 4.

With our earlier success in prehydrolysis of coniferous wood in the laboratory[16], a series of runs were made in a large scale Stake II reactor

at Université de Sherbrooke[18], softwood chips (9 x 13 x 3 mm) were fed at the rate of 1500 kg/h to give 2 min residence time. Direct steam and SO_2 gas were added, steam pressure being controlled to 1.72 MPa (250 psig) and SO_2 added 2% on (dry) coniferous wood. Runs without SO_2 (autohydrolysis) were also conducted with coniferous wood and deciduous wood. The results of these runs are shown in Table 2 and 3.

The residues so obtained in all these pretreatment studies were not washed (to remove hemisugars), as is often done, but saccharified directly.

Enzyme saccharification:

Enzymatic hydrolysis of autohydrolysed and prehydrolysed residues were carried out in 500 ml shake flasks at 50°C in a constant temperature water bath, agitated at 140 rpm for 48 hrs, assisted by a drop of Triton X100, and stainless steel balls. The pH of the hydrolysate was adjusted to 4.8 with lime, the liquid/solid ratio being between 5/1 and 7/1, i.e., 12 to 16% dry solids. For both aspen and pine wood hydrolysis, the wood saccharifying enzyme system contained cellulases (Meicelase, Meiji Seika), hemicellulases (Miles), and cellobiase (Novo), along with vitamin B_{12} and trace elements as promotors[18]. For corn stover the enzyme mixture also included pectinase (Rohm and Haas) and glucoamylase (Novo)[19].

The total hydrolysates resulting from this procedure, containing both hemicellulose and cellulose sugars, were filtered and used as the fermentation substrate. The total yield of sugars (prehydrolysed +

enzymatic hydrolysed) per 100 g oven dry original wood was chosen as an important parameter indicating the effectiveness of pretreatment.

Micro-organisms:

A pentose and wood sugars fermenting strain of yeast, P. stipitis CBS 5776, was adapted to wood hydrolysate by semi-aerobic cultivation for 24 hrs, centrifuging and recycling the yeast many times. The so called 'recycled' strain or R strain of P. stipitis[13] so obtained was maintained on YMP agar with 2% xylose as the carbon source.

Ethanol fermentation:

Inoculum of P. stipitis R was prepared in YMP medium containing 40 $g\ell^{-1}$ glucose:xylose sugars in the proportion 70:30, in a 2 ℓ shake flask on a rotary shaker at 150 rpm and 30°C. Cells were harvested by centrifugation, washed twice with saline and inoculated in the fermentation at 14 g cell dry weight per liter. Urea and diammonium phosphate were added as nitrogen and phosphorus sources at 0.1 and 0.2 $g\ell^{-1}$ respectively. All fermentations were performed under semiaerobic conditions at 30°C for 48-72 hrs[19].

Analysis:

Individual wood sugars were analysed by Waters Sugar Analyzer 1 HPLC using Biorad's Aminex HPX-87P column, with water at 65°C as the mobile phase, flow rate 0.5 ml/min and Waters 401 RI refractive index detector. The total oligomer content in the hemicellulose extract was determined by

post hydrolysis with 4% H_2SO_4 for 1 hr at 120°C before sugar analysis. Ethanol was determined by gas chromatography.

RESULTS

Auto and prehydrolysis of hardwood, softwood and corn stover

Autohydrolysis was effective in hydrolysing the hemicellulose of aspen but not of softwood. In the case of aspen, 81% of the theoretical yield of non-glucose sugars was obtained on autohydrolysis. In the presence of SO_2, the yield was raised to over 91% of theoretical yield with only 5.6% of soluble hemicellulose sugars occurring as oligomers compared to 25.5% soluble sugars as oligomers during autohydrolysis (Table 2). In the case of softwood, the presence of 2% SO_2 on wood during steam treatment resulted in excellent hydrolysis of hemicellulose, about 89% of theory, and nearly all the hemisugars appeared as monomers. Autohydrolysis with steam was less effective than SO_2 prehydrolysis, only 60% of hemisugars being recovered as soluble carbohydrates, and more than 40% of these were oligomers (Table 3). The primary effect of SO_2 addition during steam pretreatment is to increase H^+ concentration which catalyses the hydrolysis and solubilization of hemicellulose. The pH is not lowered drastically as with other mineral acids and thus restricts excessive carbohydrate degradation. Table 4 shows the results of SO_2 catalysed prehydrolysis of corn stover. As expected these results show that a maximum of about 80% hemicellulose solubilization takes place, yielding most sugars as monomers.

Enzymatic hydrolysis:

Table 5 presents the yields of wood sugars obtained on enzymatic saccharification of hardwood and softwood after auto and prehydrolysis and from corn stover after prehydrolysis with SO_2.

In all the runs, lignocellulosics steamed with 2-3% SO_2 on fiber showed the highest enzymatic accessability. The yield was over 90% of theoretical after 48 h hydrolysis of prehydrolysed aspen. With softwood the use of 2% SO_2 at 250 psig, 208°C for 2 min, resulted in 70% of wood being recovered as sugars. The maximum recovery which can be obtained from this wood based on its chemical composition is 77%, so this result is 91% of theory, which is probably the highest yet reported from coniferous wood. The use of steam alone results in the recovery of only 32% of wood as sugars from softwood and 61% from hardwood.

Enzyme saccharification of SO_2-prehydrolysed corn stover resulted in a yield of 65 g total sugars/100 g fiber, that is 86.5% of the potential sugars in corn stover based on chemical composition.

Ethanol fermentation:

Upon fermentation with the pentose fermenting yeast *P. stipitis* R, more than 90% of the sugars were utilized, with ethanol yields all within the narrow range of 0.43 to 0.45 g ethanol/g sugar consumed, this being 90% of the theoretical efficiency of ethanol formation from these sugars. With coniferous wood, ethanol concentration was 55 $g\ell^{-1}$ which is 7% by volume, a good value for economic distillation. The yield of ethanol was 372 ℓ ethanol/tonne of wood, being 78% of the theoretical maximum based on wood

composition, which calculates to be 478 ℓ/tonne. From aspen and corn stover, 346 ℓ/tonne and 388 ℓ/tonne, respectively, were obtained. These yields are 74% of theoretical. The results (Table 5) also show what happens when SO_2 is omitted and steam only (autohydrolysed) feed stock of soft and hard wood are used. With conifers the best yield of alcohol was 166 ℓ/tonne, being less than half obtained in the presence of SO_2. Yields of ethanol from autohydrolysed hard wood were much better, being 320 ℓ/tonne, not far below the 346 ℓ/tonne obtained with SO_2 prehydrolysis. Table 6 summarizes the yields of sugars and ethanol from lignocellulosics prehydrolysed by SO_2 and then enzymatically saccharified.

DISCUSSION

Addition of a small amount of SO_2 as catalyst in the 1st stage steam pretreatment of lignocellulosics, results in almost complete hydrolysis of hemicellulose to monomeric sugars, whereas with autohydrolysis of coniferous wood only about 55 to 60% hemicellulosic sugars were recovered and mostly as oligomers. With aspen, SO_2 prehydrolysis increased the proportion of solubilized hemicellulose sugars which occur as monomers. The products of SO_2 prehydrolysis are readily saccharified by enzymes as seen from the total sugar yields (Table 5), compared to autohydrolysed material[17]. The results also show that coniferous wood can be made as amenable to enzymatic hydrolysis by steam as hardwood, which has previously been considered the only viable substrate for steam pretreatment. The main difference between the treatment of hardwood and softwood is the requirement for gaseous SO_2 as an acid catalyst.

There are important benefits of SO_2 as prehydrolysing catalyst particularly as compared to H_2SO_4, which has been an industrial standard to date[20]. SO_2 as a gas distributes better in wood, can be used ahead of or with the steam, is of lower cost and is required in smaller amounts, thereby lowering the cost of neutralization before fermentation. Stainless steels which fail with H_2SO_4[21], are resistant to SO_2. Lignin may be better preserved. Equipment such as the Stake reactor, which can receive and process wood chips at high pressure in the absence of added water[18], was found to be quite satisfactory for the SO_2 prehydrolysis of coniferous wood chips and can be expected to perform well without difficulty using other feedstocks such as sawdust, and agricultural residues like straw, corncobs, stover and bagasse.

A system of commercial enzymes - cellulases, hemicellulases and cellobiase - worked successfully on the SO_2 pretreated material without any washing out of hemicellulose sugars and this resulted in relatively concentrated solutions of fermentable substrate. There was no evidence of toxicity or inhibition of the enzymes or of the P. stipitis R which together resulted in an ethanol yield of better than 70% of that theoretically possible on the basis of sugar content of the original feed stocks. Also, the efficient utilization of wood sugars by the pentose fermenting yeast P. stipitis R at realistic ethanol concentrations, shows the potentials of this yeast for the development of an economical fermentation process from biomass.

A considerable number of products that could be used in chemical industries using the present reported technology are compiled in Figure 1.

The whole process seems very simple and elegant and offers the combination of traditional fermentation know-how with new technologies, a low cost raw material, and an attractive profitable product like fuel. Also, the elimination of washing steps before enzymatic saccharification minimizes losses and saves equipment and recovery costs, and the lignin mostly being unreacted, is better preserved in quality and can be recovered in its nearly native form at good concentration and yields for higher value applications. Lastly, the capability of utilizing a wide variety of carbohydrate sources as raw materials allows this process to be tuned to local raw material availability and the application of the product ethanol in that same locality (e.g., agricultural and forest waste residues for agricultural and forest industries). The only major problem is the high present cost of the wood saccharifying enzyme system. However, there are optimistic reports that enzymatic hydrolysis may become viable in the near future[22].

CONCLUSIONS

These results suggest that to obtain a highly digestible lignocellulosic substrate from hard wood, softwood, or corn stover, it is necessary to solubilize about 90% of the hemicellulose carbohydrates during steam pretreatment. This task, as demonstrated in the present work, is made quite simple by introducing the gaseous catalyst SO_2. This treatment seems essential for coniferous wood and highly desirale for hardwoods and crop residues. The good yields of ethanol obtained on fermentation of combined pentose and hexose sugars of lignocellulosics prehydrolysed by SO_2

and enzymatically saccharified, demonstrate that such a process has industrial potential as it combines an attractive product with flexible raw material and a robust fermentation process.

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REFERENCES

1 Schoutens, G.H., Groot, J.W., and Hoebeek, J.B., Process Biochemistry, 1986, 21(1), 30

2 Faust, U., Prave, P., and Schlingmann., Process Biochemistry, 1983, 18(3), 31.

3 Sitton, O.C., Foutch, G.L., Book, N.L., and Gaddy, J.L., Process Biochemistry, 1979, 14(9), 7.

4 Wayman, M., in "Alcohol from cellulosics: The Autohydrolysis Extraction Process", Proceedings IV International Alcohol Fuels Technology Symposium, Gauruja, Brazil, 1982, Vol. 1, pp. 79-89.

5 Wayman, M., Proceedings, International Symposium on Ethanol from Biomass, Royal Society of Canada, Winnipeg, October 1982.

6 Magee, R.J. and Kosaric, N., in "Bioconversion of Hemicellulose", Advances in Biochemical Engineering and Biotechnology, 1985, 32, 61.

7 Ladish, M.R., Process Biochemistry, 1979, 14, 21.

8 Buchnolz, K., Puls, J., Godelmann, B., and Dietrichs, H.H., Process Biochemistry, 1981, 16(1), 37.

9 Schneider, H., Wang, P.Y., Chan, Y.K., and Meleszka, R., Biotechnol. Lettrs., 1981, 3, 89.

10 Du Preez, J.C. and Prior, B.A., Biotechnol. Lettrs., 1985, 7, 241.

11 Du Preez, J.C., Bosch, M., and Prior, B.A., Enzyme Microbial Techn., 1986, 8, 360.

12 Parekh, S.R. and Wayman, M., Biotechnol. Lettrs., 1986, 8, 597.

13 Parekh, S.R., Yu, S., and Wayman, M., Appl. Microbiol. Biotechnol., 1986, in press.

14 Communication, Oil and Gas Journal, 1984, p. 6.

15 Communication, Chemistry and Industry, 1984, p. 14.

16 Wayman, M., Tallevi, A., and Winsboroow, B., Biomass, 1984, 6, 183.

17 Puls, J., Poutanen, K., Korner, H.U., and Viikari, L., Appl. Microbiol. Biotechnol., 1985(6), 416.

18 Wayman, M., Parekh, S., Chornet, E., and Overend, R., Biotechnol. Lettrs., 1986, 8, 749.

19 Wayman, M., and Parekh, S., Biotechnol. Lettrs., 1985, 7, 909.

20 Burton, R.J., "New Zealand wood hydrolysis process". In Proceedings, International Symposium on Ethanol from Biomass, Royal Society of Canada, Winnpieg, 1982, pp. 247-270, October.

21 Mackie, K., Deverell, K., and Callender, I., "Aspects of wood hydrolysis via the Dilute Sulphuric Acid Process", in ref. 20, 1982, pp. 271-311.

22 Pourquie, J., and Glikmans, G., "Steam explosion and enzymatic hydrolysis of lignocellulosic material". In VII International Symposium on Alcohol Fuels, October 1986, Paris, France.

Table 1

Summative Analysis of Softwood, Hardwood and Corn Stover.

	% of dry weight		
	Softwood[a]	Hardwood[b]	Corn stover
Total polysaccharides	70.0	68.0	68.3
Klason lignin	26.0	20.0	15.2
Acid soluble lignin	0.6	1.33	1.0
Extractives	4.4	2.86	9.5
Ash	-	-	2.0
Other	-	7.81	4.0
Total	100.0	100.0	100.0
Polysaccharides			
Glucose	48	43	53.3
Non-glucose	22	25	15.0

a coniferous wood, mixed pine and spruce chips from eastern township of Quebec

b deciduous wood, aspen poplar chips

Table 2

Chemical Characterization of the Water-Extracted Fraction after Auto and Prehydrolysis of Deciduous Wood.

Pretreatment conditions	Glucose (%)	Total sugars (%)	Fiber residue
Autohydrolysis (steam) 1.72 MPa (250 psig), 2 min			
Monomer	2.35	16.15	72.06
Oligomer	0.85	9.25	
Total	3.20	25.40	
% of theory			
Glucose	6.8		
Non-glucose	81		
Prehydrolysis, 3% SO_2 on wood, 75 psig, 30 min			
Monomer	2.89	27.08	69.9
Oligomer	0.60	1.61	
Total	3.49	28.69	
% of Theory			
Glucose	7.4		
Non-glucose	91.6		

Table 3

Chemical Characterization of the Water-Extracted Fraction after Auto and Prehydrolysis of Coniferous Wood.

Pretreatment conditions	Glucose (%)	Total sugars (%)	Fiber residue
Autohydrolysis (steam) 1.72 MPa (250 psig), 2 min			
Monomer	2.24	11.33	75.6
Oligomer	1.93	7.54	
Total	4.17	18.87	
% of theory			
Glucose	7.8		
Non-glucose	60		
Prehydrolysis, 2% SO_2 on wood, at 1.72 MPa (250 psig), 2 min			
Monomer	5.41	26.56	71.3
Oligomer	0.14	0.54	
Total	5.55	27.10	
% of Theory			
Glucose	10.5		
Non-glucose	89.4		

Table 4

Chemical Characterization of the Water-Extracted Fraction after Prehydrolysis of Corn Stover.

Pretreatment conditions	Glucose (%)	Total sugars (%)	Fiber residue
Prehydrolysis, 3% SO_2 on fiber, 30 min, 75 psig (160°C)			
Monomer	7.82	20.67	71.14
Oligomer	0.93	1.08	
Total	8.75	21.75	
% of theory			
Glucose	15.0		
Non-glucose	79		

Table 5

Fermentation to Ethanol of Combined Hemicellulose and Cellulose Hydrolysate of Softwood, Hardwood and Corn Stover by *P. stipitis* R.

Pretreatment conditions	Sugar yield after enzymatic hydrolysis		Yields after fermentation				
	total sugars ($g\ell^{-1}$)	% on raw material	reducing sugars ($g\ell^{-1}$)	Ethanol ($g\ell^{-1}$)	g ethanol/ g sugar consumed	% ethanol on fiber	ℓ ethanol/ dry tonne of raw material
a) *softwood* (pine)							
SO_2-prehydrolysed fiber	134	71.7	6	55	0.43	29	372
steam autohydrolysed fiber	36	32	3	15.1	0.45	13	166
b) *hardwood* (aspen)							
SO_2-prehydrolysed fiber	123	68	5.6	52	0.44	27	346
steam autohydrolysed fiber	72	61	6	30	0.45	25	320
c) *corn stover*							
SO_2-prehydrolysed fiber	120	65	6	52	0.44	30.2	388

Table 6

Summary on Yield of Sugars and Ethanol from Lignocellulosics Prehydrolysed by SO_2.

	Hardwood	Softwood	Corn stover
SO_2 prehydrolysis:			
hemi sugars, kg/tonne	252	219	130
% theoretical yield*	91.6	89.4	79
Enzyme hydrolysis:			
total sugars, kg/tonne	680	717	650
% theoretical yield*	91.2	95	86.5
Ethanol:			
L/tonne	346	372	388
% theoretical yield*	73.6	76.8	81

* based on chemical composition:

hemicellulose sugars, kg/tonne	275	245	165
total sugars, kg/tonne	745	755	752
ethanol, L/tonne	470	478	480

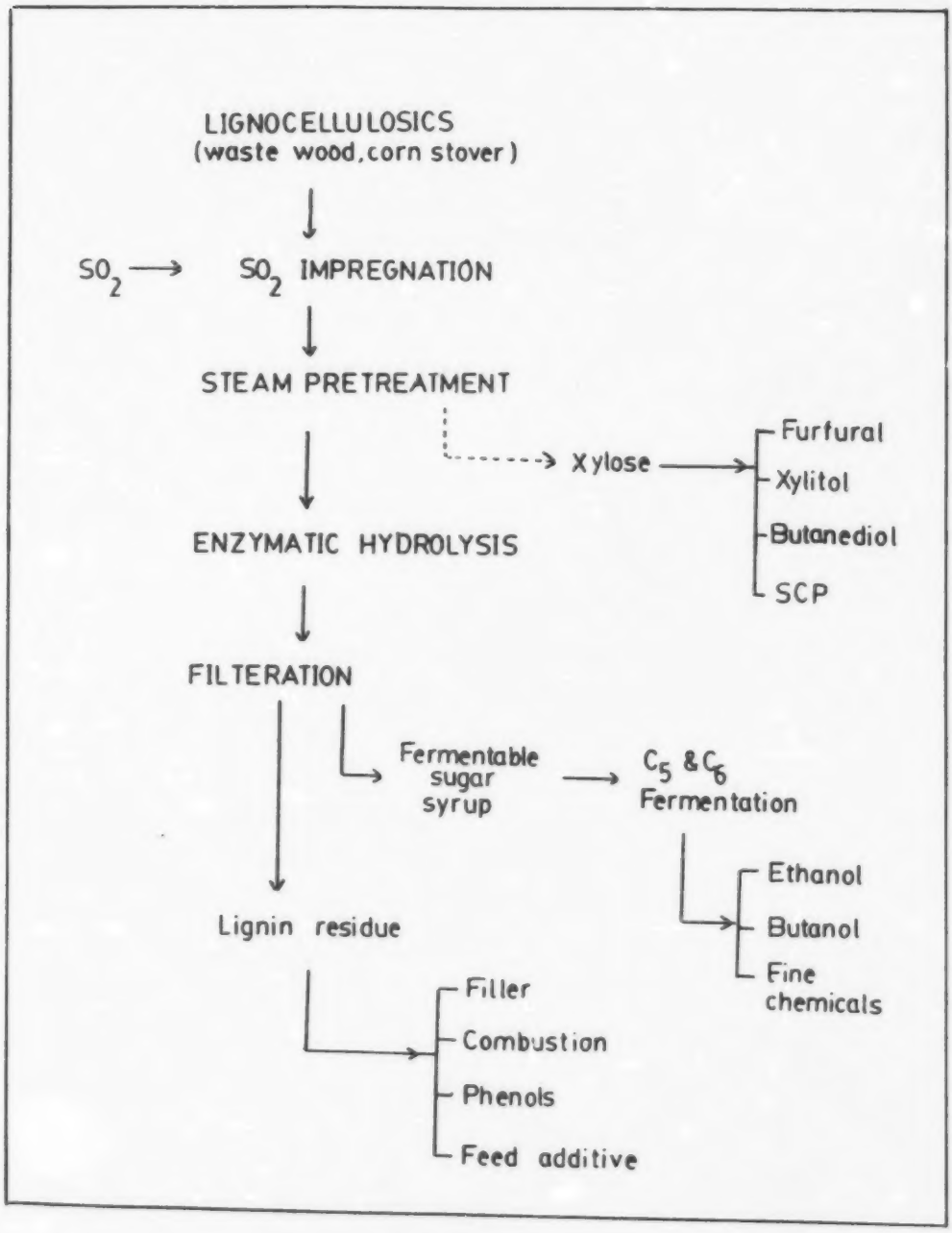

Figure 1. Products from lignocellulosics, enzymatically saccharified after prehydrolysis by SO_2.

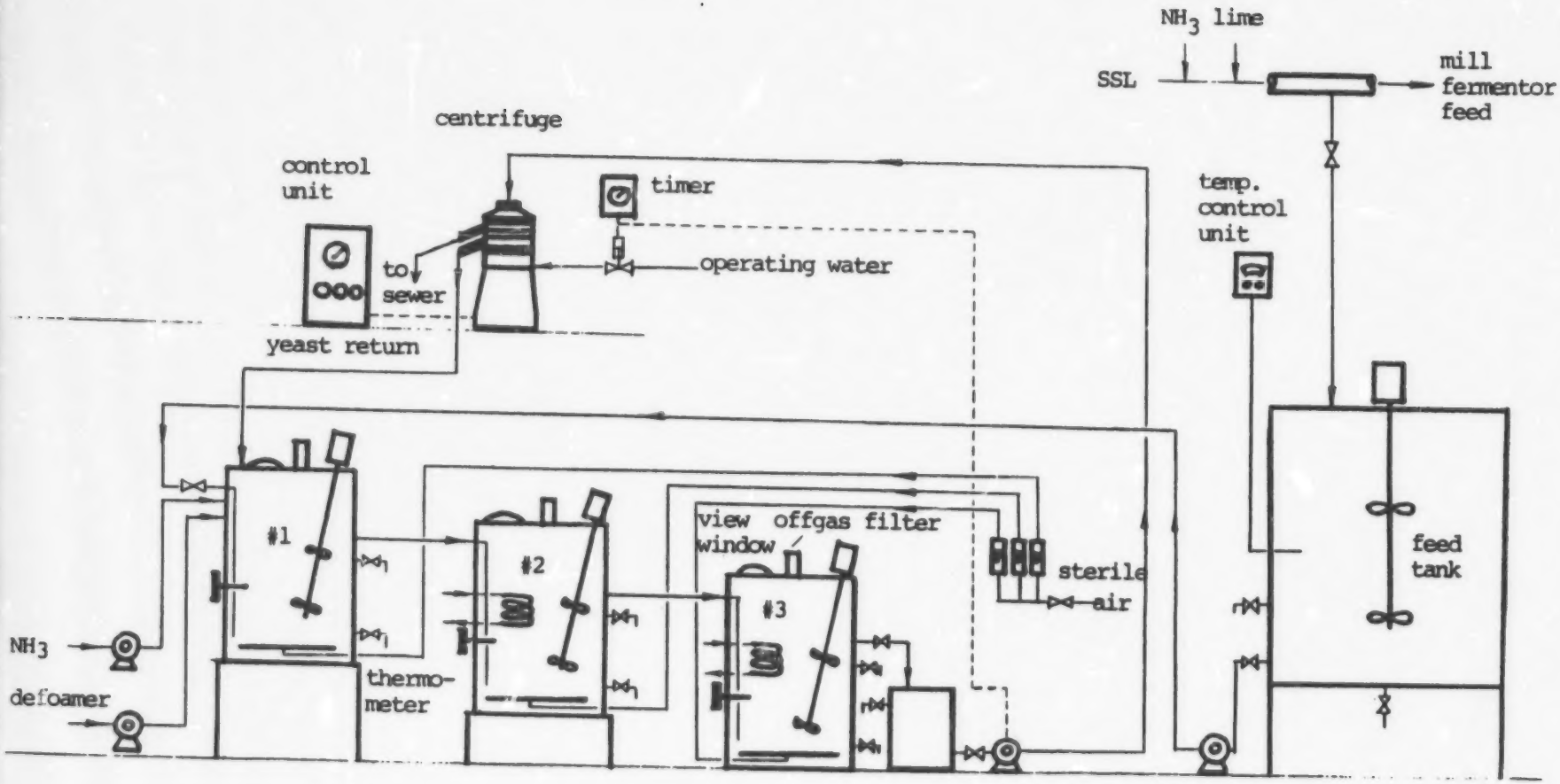

Fig. 2. Schematic Diagram of the 3-Stage Continuous Fermentation System

fermentors:
dia. 31''
ht. 48''
ht. to overflow 32''
liquid-holding vol. 100 gal.

feed tank:
dia. 57''
ht. 67''
cap. 550 gal.

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